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(54) Title: HMG2 PROMOTER EXPRESSION SYSTEM AND POST-HARVEST PRODUCTION OF GENE PRODUCTS IN PLANTS AND PLANT CELL CULTURES

(57) Abstract

The promoter elements of plant HMG2 HMGR genes are described. The HMG2 promoter elements are responsive to pathogen-infection, pest-infestation, wounding, or elicitor or chemical treatments. The HMG2 elements are also active in specialized tissues of the plant including pollen and mature fruits. HMG2 promoter elements and HMG2-derived promoters can be advantageously used to drive the expression of disease and pest resistance genes, whereby transgenic plants containing such gene constructs would be resistant to the targeted disease and pest. In particular, the HMG2 gene expression system can be utilized in developing nematode resistant plants. HMG2 promoter elements and HMG2-derived promoters can also be advantageously used to drive the post-harvest expression of desired gene products in plants, wherein the expression of the desired gene product is deferred until the expression is induced by mechanical wounding and/or elicitor treatment of all plant tissue shortly before, during or shortly after harvesting the plant. Further, HMG2 promoter elements and HMG2-derived promoters can also be advantageously used to drive the expression of desired gene products in plant cell cultures, wherein the expression of the desired gene product is deferred until the expression is induced by mechanical wounding or elicitor treatment.

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HMG2 PROMOTER EXPRESSION SYSTEM AND POST-HARVEST PRODUCTION OF GENE PRODUCTS IN PLANTS AND PLANT CELL CULTURES

This application is a continuation-in-part of co-pending application Serial No. 08/100,816 filed August 2, 1993, which is hereby incorporated by reference in its entirety.

1. INTRODUCTION

The present invention relates in part to *HMG2* promoters, active fragments thereof, and their use to drive the expression of heterologous genes. Expression vectors may be constructed by ligating a DNA sequence encoding a heterologous gene to an *HMG2* promoter or a promoter modified with an *HMG2* cis-regulatory element. Such constructs can be used for the expression of proteins and RNAs in plant cell expression systems in response to elicitor or chemical induction, or in plants in response to wounding, pathogen infection, pest infestation as well as elicitor or chemical induction.

The present invention also relates to a method of producing gene products in harvested plant tissues and cultures. The "post-harvest" production method utilizes tissues or cultures from transgenic plants or plant cells, respectively, engineered with expression constructs comprising inducible promoters operably linked to sequences encoding desirable protein and RNA products. Treatment of the harvested tissues or cultures with the appropriate inducer and/or inducing conditions initiates high level expression of the desired gene product. The post-harvest production method may advantageously be used to produce gene products and metabolites of gene products that are labile, volatile or toxic or that are deleterious to plant growth or development.

This aspect of the invention is demonstrated by way of examples which describe the production of transgenic plants engineered with expression constructs comprising inducible promoters operably linked to coding sequences of interest, and

which show that days-old and weeks-old harvested tissues from such transgenic plants remain capable of induced production of high levels of the gene product of interest.

2. BACKGROUND OF THE INVENTION

Plant diseases caused by fungal, bacterial, viral and nematode pathogens cause significant damage and crop loss in the United States and throughout the world. Current approaches to disease control include breeding for disease resistance, application of chemical pesticides, and disease management practices such as crop rotations. Recent advances in plant biotechnology have led to the development of transgenic plants engineered for enhanced resistance to plant pests and pathogens. These successes indicate that biotechnology may significantly impact production agriculture in the future, leading to improved crops displaying genetic resistance. This mechanism of enhanced disease resistance has the potential to lower production costs and positively impact the environment by reducing the use of agrichemicals.

Recent advances in plant biotechnology have led also to the development of transgenic plants engineered for expression of new functions and traits. Such engineering has produced transgenic plants having new synthetic capabilities. These successes indicate that biotechnology may enhance the role of plants as a producer of chemicals, pharmaceuticals, polymers, enzymes, etc. The engineering of novel synthetic capabilities has the potential to increase crop values as well as create new uses for crops such as tobacco.

2.1. PLANT DEFENSE RESPONSES

Higher plants have evolved a variety of structural and chemical weapons for protection against pathogens, predators, and environmental stresses. Expression of resistance toward pathogens and pests involves the rapid induction of genes leading to accumulation of specific defense-related compounds (Dixon et al., 1990, Adv. Genet. 28:165-234). These responses include accumulation of

phytoalexin antibiotics, deposition of lignin-like material, accumulation of hydroxyproline-rich glycoproteins; and increases in hydrolytic enzymes such as chitinase and glucanase.

Phytoalexins are low molecular weight compounds which accumulate as a result of pathogen challenge. Phytoalexins have antimicrobial activity and have proved to be of critical importance in disease resistance in several plant:pathogen interactions (Darvill et al., 1984, Annu. Rev. Plant Physiol. 35:243; Dixon et al., 1990, Adv. Genet. 28:165-234; and Kuc and Rush, 1895, Arch. Biochem. Biophys. 236:455-472). Terpenoid phytoalexins represent one of the major classes of plant defense compounds and are present across a wide range of plant species including conifers, monocots and dicots. The regulation of terpenoid phytoalexin biosynthesis has been most extensively studied among the solanaceae (e.g., tomato, tobacco, potato), however, most important crop species utilize this pathway in disease resistance.

In well characterized plant:microbe interactions, resistance to pathogens depends on the rate at which defense responses are activated (Dixon and Harrison, 1990, Adv. Genet. 28:165-234). In an incompatible interaction, the pathogen triggers a very rapid response, termed hypersensitive resistance (HR), localized to the site of ingress. In the susceptible host, no induction of defense responses is seen until significant damage is done to host tissue.

Defense-responses, including phytoalexin accumulation, can also be induced in plant cell cultures by compounds, termed "elicitors", for example components derived from the cell walls of plant pathogens (Cramer et al., 1985, EMBO J. 4:285-289; Cramer et al., 1985, Science 227:1240-1243; Dron et al., 1988, Proc. Natl. Acad. Sci. USA 85:6738-6742). In tobacco cell cultures, fungal elicitors trigger a coordinate increase in sesquiterpene phytoalexin biosynthetic enzymes and a concomitant decrease in squalene synthetase which directs isoprene intermediates into sterol biosynthesis (Chappell and Nable, 1987, Plant Physiol. 85:469-473; Chappell

et al., 1991, Plant Physiol. 97:693-698; Threlfall and Whitehead, 1988, Phytochem. 27:2567-2580).

2.2. PHYSIOLOGICAL ROLES OF HMGR IN PLANTS

A key enzyme involved in phytoalexin biosynthesis and, hence, plant defense against diseases and pests, is 3-hydroxy-3-methylglutaryl CoA reductase (HMGR; EC 1.1.134). HMGR catalyzes the conversion of 3-hydroxy-3-methylglutaryl CoA to mevalonic acid. Mevalonic acid is an intermediate in the biosynthesis of a wide variety of isoprenoids including defense compounds such as phytoalexin.

In addition to phytoalexin biosynthesis, the mevalonate/isoprenoid pathway produces a diverse array of other biologically important compounds (see Figure 1). These include growth regulators, pigments, defense compounds, UV-protectants, phytotoxins, and specialized terpenoids such as taxol, rubber, and compounds associated with insect attraction, fragrance, flavor, feeding deterrence and allelopathy. Many of these compounds have critical roles in cellular and biological functions such as membrane biogenesis, electron transport, protein prenylation, steroid hormone synthesis, intercellular signal transduction, photosynthesis, and reproduction.

The conversion of 3-hydroxy-3-methylglutaryl CoA to mevalonic acid appears to be the rate-limiting step of isoprenoid biosynthesis. Thus, the regulation of HMGR expression serves as a major control point for a diversity of plant processes including defense responses. Plant HMGR levels change in response to a variety of external stimuli including light, plant growth regulators, wounding, pathogen attack, and exogenous sterols (Brooker and Russell, 1979, Arch. Biochem. Biophys. 198:323-334; Russell and Davidson, 1982, Biochem. Biophys. Res. Comm. 104:1537-1543; Wong et al., 1982, Arch. Biochem. Biophys. 216:631-638 and Yang et al., 1989, Mol. Plant-Microb. Interact. 2:195-201) and vary dramatically between tissues and developmental stages of plant growth (Bach et al., 1991, Lipids 26:637-648 and Narita and

Gruissem, 1991, J. Cell. Biochem 15A:102 (Abst)). Reflecting this complexity, multiple HMGR isozymes exist in plants which are differentially regulated and may be targeted to distinct organellar locations. In addition to microsomal locations, plant HMGR isozymes have been localized to mitochondrial and chloroplast membranes (Brooker and Russell, 1975, Arch. Biochem. Biophys. 167:730-737 and Wong et al., 1982, Arch. Biochem. Biophys. 216:631-638).

2.3. PLANT HMGR GENES

HMGR genomic or cDNA sequences have been reported for several plant species including tomato (Narita and Gruissem, 1989, Plant Cell 1:181-190; Park, 1990, *Lycopersicon esculentum* Mill. Ph.D. Dissertation, Virginia Polytechnic Institute and State University, Blacksburg, VA), potato (Choi et al., 1992, Plant Cell 4:1333-1344 and Stermer et al., 1991, Physiol. Mol. Plant Pathol. 39:135-145), radish (Bach et al., 1991, American Oil Chemists Society, Champaign, IL page 29-49), *Arabidopsis thaliana* (Caelles et al., 1989, Plant Mol. Biol. 13:627-638; Learned and Fink, 1989, Proc. Natl. Acad. Sci. USA 86:2779-2783), *Nicotiana sylvestris* (Genschik et al., 1992, Plant Mol. Biol. 20:337-341), *Catharanthus roseus* (Maldonado-Mendoza et al., 1992, Plant Physiol. 100:1613-1614), the *Hevea* rubber tree (Chye et al., 1991, Plant Mol. Biol. 16:567-577) and wheat (Aoyagi et al., 1993, Plant Physiol. 102:622-638). Cloning of additional HMGR genes from rice (Nelson et al., 1991, Abstract 1322, 3rd Intl. Cong. Intl. Soc. Plant Mol. Biol., Tucson, AZ), and tomato (Narita et al., 1991, J. Cell. Biochem 15A:102 (Abst)) also has been reported but no DNA sequence information has been published for these HMGR genes. In all plants thus far investigated, HMGR is encoded by a small gene family of two (*Arabidopsis*) or more members (tomato, potato, *Hevea*). See Table 1 for a summary of published information pertaining to plant HMGR genes.

TABLE 1: SUMMARY OF CLONED PLANT HMGR SEQUENCES AND THEIR REGULATION. (Footnotes and references on next page)

Species	HMGR gene	Description[a]	Regulation constit.	pollen	wound	pathogen[b]	other	Promoter[c] Analyses	Ref.
Lycopersicon esculentum	hmg2	G, cDNA, F, S	--	+	+	B,F,E,N	mature fruit	S, GUS, D	1
(tomato)	hmg1	cDNA, P, S	low	?	?	-	immat. fruit	-	1,2
Solanum	hmg1	cDNA, F, S	low	?	+	--	elic. suppressed	GUS	3,4
tuberosum	hmg2	cDNA, P, S	-	?	+	B,E	-	-	3
(potato)	hmg3	cDNA, P, S	-	?	+	B,E	-	-	3
Nicotiana sylvestris	hmgr	cDNA, F, S	+	?		E,V	roots, protoplasts	-	5
Arabidopsis	hmg1	cDNA, G, F, S	low	?	?	?	-	-	6
thaliana	hmg2	cDNA	-	?	?	?	-	-	7
Hevea	hmg1	G, cDNA, F, S	-	?	?	?	laticifer, ethylene ind	-	8
brasiliensis	hmg2	cDNA, P, S	?	?	?	?	-	-	9
	hmg3	cDNA, F, S	+				"housekeeping"	S	8
Catharanthus roseus	hmgr	cDNA, F, S	+	?	?	?	-	-	10
Raphanus sativus	hmg1	cDNA, F, S	?	?	?	?	-	-	11
(radish)	hmg2	cDNA, F, S	?	?	?	?	isolated seedlings	-	
Triticum	hmgr10	cDNA, P	low	?	-	?	callus	-	12
aestivum	hmgr18	cDNA, P	+	?	-	?	callus	-	
(wheat)	hmgr23	cDNA, P	-	?	-	?	roots, callus	-	

TABLE 1 (continued)

Footnotes

a. G, genomic clone; cDNA, complementary DNA generated from mRNA (lacks promoter, introns); F, full-length clone (contains entire coding sequence); P, partial sequence; GenEMBL accession numbers are listed

b. Induced by bacterial pathogen (B), fungal pathogen (F), parasitic nematode (N), or defense elicitors (E).

c. Promoter has undergone sequence analysis (S), has been analyzed in transgenic plants by expression of promoter:reporter gene fusions (GUS), has undergone deletion analyses (D).

References and GenEMBL Accession Numbers[a]

1. This disclosure and [M63642]
2. Narita et al, 1989, Plant Cell 1:181-190.
3. Choi, et al., 1992, Plant Cell 4:1333-1344. [L01400; L01401; L01402]
4. Stermer et al., 1992, Phytopathology 82:1085 (Abstract).
5. Genschik et al., 1992. Plant Mol. Biol. 20:337-341. [X63649]
6. Learned and Fink, 1989, Proc. Natl. Acad. Sci. USA 86:2779-2783. [J04537]
7. Caelles et al., 1989, Plant Mol. Biol. 13:627-638. [X15032]
8. Chye et al., 1991, Plant Mol. Biol 16:567-577. [X54657; X54658; X54659]
9. Chye et al., 1992, Plant Mol. Biol. 19:473-484.
10. Maldonado-Mendoza et al., 1992, Plant Physiol. 100:1613-1614. [M96068]
11. Bach et al., 1991, In Physiology and Biochemistry of Sterols (Patterson,G. and W. Nes, eds.), Am. Oil Chemists Soc., Champaign, IL, pp. 29-49.
12. Aoyagi et al., 1993, Plant Physiol. 102:623-628

[a] GenEMBL Accession Number are in brackets.

2.4. TOMATO HMGR GENES

Tomato HMGR genes have been isolated by direct probing of a tomato genomic library with yeast *HMG1* cDNA sequences (Cramer et al., 1989, J. Cell. Biochem. 13D:M408; Park, 1990, *Lycopersicon esculentum* Mill. Ph.D. Dissertation, Virginia Polytechnic Institution and State University, Blacksburg, VA). One of these genes, designated tomato *HMG2*, has been characterized further and the sequence of the coding region reported (Park et al., 1992, Plant Mol. Biol. 20:327-331; GenBank M63642). The nucleic acid sequence of tomato *HMG2* has been compared to those of other plant HMGRs. Sequence comparisons within regions encoding the HMGR protein show identities in the range of 69 to 96%, with the most closely related sequences being a partial cDNA sequence of potato *HMG2* (96% sequence identity for 744 base at the C-terminus coding region) and an HMGR cDNA from *Nicotiana sylvestris* (89% sequence identity; entire coding region). The tomato *HMG2* gene is distinct from tomato *HMG1* showing 78% sequence identity within the region encoding the N-terminus (1067 bases compared; see Figure 2). Comparisons of sequences outside the coding region (e.g., the 5'-untranslated leader sequence) reveal very little sequence conservation between divergent species and only limited homology among HMGR genes within the Solanaceae. Tomato *HMG2* shows less than 75% sequence identity over an 81 base stretch with the *N. Sylvestris* leader sequences. Comparisons between the 5'-leader sequences of tomato *HMG1* and *HMG2* are included in Figure 2.

The coding region of the tomato *HMG2* gene is interrupted by three introns and encodes a protein with a calculated molecular mass of 64,714 (Park et al., 1992, Plant Mol. Biol. 20:327-331). Table 2 shows a comparison of the derived amino acid sequence of the tomato *HMG2* to HMGRs of other organisms. The N-terminal membrane domain of the tomato *HMG2* HMGR is significantly smaller, shows no sequence similarity to the yeast or animal transmembrane domain, and contains 1-2 potential membrane spanning regions compared to

7-8 postulated in non-plant HMGRs (Basson et al., 1988, Mol. Cell Biol. 8:3797-3808; Liscum et al., 1985, J. Biol. Chem 260:522-530). This transmembrane domain is also highly divergent among plant species and among HMGR isogenes within a species although the actual membrane spanning residues are quite conserved.

Amino acid sequence comparisons between the N-terminal two hundred residues of tomato *HMG2* HMGR and other plant HMGRs yield sequence identities in the range of 44-85% (Table 2). Tomato *HMG1* and *HMG2* show only 75% sequence identity in this region. The catalytic domain of tomato *HMG2*, located within the C-terminal 400 amino acids, shows significant sequence homology with the yeast (56% identity; 75% similarity) and human (55% identity; 75% similarity) HMGRs. This domain is highly conserved between plant species: comparisons between tomato *HMG2* HMGR (residues 200-602) and other plant HMGRs yield amino acid identities ranging from 74-90% (see Table 2).

In contrast to the information available on plant HMGR coding sequences, relatively little is known of the structure of plant HMGR promoters. Indeed, to date, only one plant HMGR promoter sequence has been reported (Chye et al., 1991, Plant Mol. Biol. 16:567-577).

Table 2. Amino acid identity (%) of the membrane and catalytic domains of plant HMGRs.

	Solanaceae					Euphorb.		Apo	Brassicaceae		
	Tom1	*Tom2*	*Pot1*	*Pot3*	*Nic*	*Hev1*	*Hev3*	*Car*	*Ara1*	*Rad1*	*Rad2*
Tom1	*100* -	*75* -	*98* -	- -	*77* -	*46* -	*53* -	*64* -	*57* -	*48* -	*57* -
Tom2		*100* *100*	*74* *90*	- *90*	*85* *95*	*44* *85*	*52* *74*	*66* *84*	*50* *81*	*48* *80*	*52* *80*
Pot1			*100* *100*	- *90*	*76* *93*	*45* *88*	*54* *85*	*63* *88*	*58* *83*	*49* *83*	*57* *83*
Pot3				- *100*	- *92*	- *86*	- *85*	- *84*	- *82*	- *81*	- *82*
Nic					*100* *100*	*42* *87*	*49* *86*	*63* *87*	*54* *83*	*49* *83*	*57* *82*
Hev1						*100* *100*	*44* *86*	*38* *87*	*76* *86*	*51* *86*	*55* *85*
Hev3							*100* *100*	*46* *85*	*56* *82*	*48* *82*	*48* *82*
Car								*100* *100*	*48* *81*	*44* *82*	*49* *82*
Ara1									*100* *100*	*73* *94*	*78* *95*
Rad1										*100* *100*	*72* *93*
Rad2											*100* *100*

Upper row: membrane domains (1 to 200 amino acids); Lower row: catalytic domain (201 to 600 amino acids). Plant species and hmgr isogene (where designated) are indicated: *L. esculentum* [tomato *hmg1* (incomplete sequence) and *hmg2*; *Tom1, Tom2*], *S. tuberosum* [potato; *Pot1, Pot3* (incomplete seq.)], *N. sylvestris* (*Nic*, isogene unknown), *A. thaliana hmg*1 (*Ara*1), *H. brasiliensis* (rubber tree; *Hev*1, *Hev*3), *C. roseus* (*Car*, isogene unknown), and *R. sativus* (radish; *Rad1, Rad2*). Comparisons are based on the sequences available from GenBank.
Euphorb.: Euphoriaceae, Apo.: Apocynaceae.

2.5 THE FUNCTIONS AND EXPRESSION PATTERNS OF DIFFERENT HMGR GENES

Specific plant HMGR isogenes show very distinct patterns of regulation during development and in response to pathogen infection or pest infestation (Caelles et al., 1989, Plant Mol. Biol. 13:627-638; Choi et al., 1992, Plant Cell 4:1333-1344; Chye et al., 1992, Plant Mol. Biol. 19:473-484; Narita and Gruissem, 1989, Plant Cell 1:181-190; Park et al., 1992, Plant Mol. Biol. 20:327-331; Park, 1990, *Lycopersicon esculentum* Mill. Ph.D. Dissertation, Virginia Polytechnic Institute and State University, Blacksburg, VA; Yang et al., 1991, Plant Cell 3:397-405).

In the *Solanaceae*, the pathogen-induced synthesis of phytoalexin antibiotics is due to the induction of microsome-associated HMGR (Shih and Kuc, 1973, Phytopathology 63:826-829 and Stermer and Bostock, 1987, Plant Physiol. 84:404-408). In cultured cells, the induction occurs with the addition of elicitors isolated from the cell walls of pathogenic microbes (Chappell et al., 1991, Plant Physiol. 97:693-698 and Stermer and Bostock, 1987, Plant Physiol. 84:404-408). Typically, elicitor treatment results in a marked, transient increase in HMGR mRNA level that is typical of transcriptionally regulated defense genes in plants (Cramer et al., 1985, EMBO J. 4:285-289; Dixon and Harrison, 1990, Adv. Genet. 28:165-234; Yang et al., 1991, Plant Cell 3:397-405).

The rapid pathogen-induced HMGR expression appears to be encoded by the *HMG2* HMGR isogene. This has been established in tomato cells treated with elicitors (Park, 1990, *Lycopersicon esculentum* Mill. Ph.D. Dissertation, Virginia Polytechnic Institute and State University, Blacksburg, VA) and in potato tubers infected with the soft-rotting bacterium *Erwinia carotovora* (Yang et al., 1991, Plant Cell 3:397-405). In potato tuber, *HMG2* mRNA levels increased 20-fold within 14 hr following bacteria inoculation but was not induced by wounding in the absence of pathogen (Yang et al., 1991, Plant Cell 3:397-405).

HMG2 expression has been shown to be critical to disease resistance. An elicitor, arachidonic acid, induces defense responses including increases in *HMG2* mRNA and thus, phytoalexin synthesis in potato tubers (Stermer and Bostock, 1987, Plant Physiol. 84:404-408 and Yang et al., 1991, Plant Cell 3:397-405). Tubers treated with arachidonic acid 48 hr prior to inoculation with *Erwinia carotovora* were completely resistant to rotting. Tubers inhibited in HMGR activity by mevinolin (an HMGR-specific competitive inhibitor) showed significantly increased rotting (Yang et al., 1991, Plant Cell 3:397-405).

Homologs to the tomato *HMG2* HMGR isogene, based on analogous expression patterns, exist in other plants. For example, potato *HMG2* and *HMG3* mRNAs also are induced in response to wounding, elicitors and bacterial pathogens (Choi et al., 1992, Plant Cell 4:1333-1344 and Yang et al., 1991, Plant Cell 3:397-405). An HMGR isogene isolated from *Nicotiana sylvestris* is induced by virus inoculation and defense elicitors (Genschik et al., 1992, Plant Mol. Biol. 20:337-341). Similarly, an HMGR isogene of rice also is induced by wounding and elicitors (Nelson et al., 1991, Abst 1322, 3rd Intl. Cong. Intl. Soc. Plant Mol. Biol., Tucson, AZ) suggesting that defense-specific *HMG2* homologs are also present in monocotyledonous plants. However, the three HMGR cDNAs isolated from wheat apparently are not wound-inducible (Aoyagi et al., 1993, Plant Physiol. 102:623-628). Although DNA sequences have not been isolated, increases in HMGR enzyme activity in response to bacterial or fungal pathogens have been documented in a number of other plant species suggesting that the *HMG2* isogene is widely represented among plants.

Tomato *HMG2* is quite distinct from the tomato *HMG1* isogene at the nucleic acid level (Figure 2). The *HMG1* HMGR isogene appears to be expressed under circumstances that are different from those that induce the expression of the *HMG2* gene. Narita and Gruissem have shown that tomato *HMG1* is expressed at low levels in all tomato tissues analyzed and is highly expressed in immature fruit during the period of rapid

growth (Narita and Gruissem, 1989, Plant Cell 1:181-190; Narita et al., 1991, J. Cell. Biochem. 15A:102 (Abst)). Thus, tomato *HMG1* may function in sterol synthesis and membrane biogenesis. Choi et al. have shown that potato *HMG1* expression is induced in potato tubers by wounding but is suppressed by defense-elicitors or bacterial pathogens (Plant Cell 4:1333-1344). These data suggest that the HMGR encoded by the *HMG1* is associated witn the biosynthetic branch responsible for sterol production. Based on similarities in sequence or expression patterns (*e.g.*, constitutive low level expression, suppression by elicitors or microbes, high expression during rapid cell division), *Hevea HMGR3*, potato *HMG1*, *Arabidopsis HMG1*, and radish *HMG2* probably belong to this *HMG1* HMGR isogene class.

2.6. PLANT HMGR PROMOTERS

Little is known of the promoters of plant HMGR genes. To date, there have been no known publications of studies analyzing the structure of plant HMGR promoters (*e.g.*, deletion analyses, DNaseI footprint analysis, DNA-protein binding/gel retardation analyses). One article (Chye et al., 1991, Plant Mol. Biol. 16:567-577) documents approximately 1.5 kb of the upstream sequence of the *Hevea HMG1* gene. This sequence shows no significant homology to the analogous region of the tomato *HMG2* promoter disclosed herein. Another report cursorily mentioned expression of potato HMGR13 (presumably an *HMG1* homolog) promoter:β-glucuronidase (GUS) fusions in transgenic tobacco (Stermer et al., 1992, Phytopathology 82:1085), but revealed no details concerning the promoter used nor the construction of the fusion.

2.7. PRODUCTION OF DESIRABLE GENE PRODUCTS IN PLANTS

The concept of using transgenic plants to produce desirable gene products is by no means new. Virtually all examples reported to date of such engineering of transgenic plants have utilized "constitutive" promoters, especially the

cauliflower mosaic virus (CaMV) 35S promoter or enhanced versions thereof, to drive production of transgenic products. The following is a representative list of the many mammalian therapeutic proteins that have been produced in plants using the CaMV 35S promoter or derivatives. Expression of mouse immunoglobulin chains was achieved by transforming tobacco leaf segments with gamma- or kappa-chain cDNAs derived from a mouse hybridoma cell line (Hiatt et al., 1989, Nature 342:76-78; Hein et al., 1991, Biotechnol. Prog. 7:455-461). Transformed plants expressing either gamma or kappa chains were genetically crossed resulting in the production of assembled, functional antibodies (termed "plantibodies"). Transgenic tobacco producing human serum albumin (Sijmons et al., 1990, Bio/Technology 8:217-221), rabbit liver cytochrome P450 (Saito et al., 1991, Proc. Natl. Acad. Sci. USA 88:7041-7045), hamster 3-hydroxy-3-methylglutaryl CoA reductase (Chappell et al., 1991, Plant Physiol. 96:127), and the hepatitis B surface antigen (Mason et al., 1992, Proc. Natl. Acad. Sci. USA 89:11745-11749) have also been reported. HSA produced as a fusion protein with plant prepro-signals resulted in a secreted HSA protein that was indistinguishable from authentic mature human protein (Sijmons et al., 1990, Bio/Technology 8:217-221). The hepatitis B antigen-expressing plants accumulated spherical HBsAg particles with physical and antigenic properties similar to human serum-derived HBsAg particles and to those generated in yeast (current source of human vaccine). Plant virus-mediated expression of human proteins in plants, including human interferon, α- and β-hemoglobin, and melanin has also been demonstrated (Fraley, R., 1992, Bio/Technology 10:36-43; de Zoeten et al., 1989, Virology 172:213-222).

The constitutive expression of desirable gene products, however, is disadvantaged whenever the desirable gene product is 1) labile, 2) deleterious to the growth or development of the transgenic plant or culture line, 3) toxic to humans, livestock, animals, or insects that could inadvertently eat the transgenic plant, 4) a real or potential

environmental hazard, or 5) a security risk due to the extremely high value of the gene product. Moreover, some constitutive promoters, such as the CaMV 35S, decline in activity as plants matures. Such losses of activity can result in inefficient exploitation of the host plant as a production system, since mature or maturing plants have greater biomass and, often, greater excess biosynthetic capacity for the production of the "extraneous" desirable gene product than younger plants which are programmed to devote much of their biosynthetic capacities to normal growth and development functions.

Compared to the aforementioned conventional approach, the post-harvest production method of the instant invention can be a much more efficient and productive means of producing desirable gene products in plants. Using the post-harvest production method, the desired gene product is not produced until after the plant tissue or cell culture has been harvested and induced. This obviates any problems that might arise from the presence of the desired gene product during the growth of the plant or culture line.

3. SUMMARY OF THE INVENTION

One aspect of the present invention relates to the use of plant promoter sequences responsive to wounding, pathogen infection, pest infestation as well as to elicitors or chemical inducers. This aspect of the invention generally relates to the use of promoter sequences of 3-hydroxymethyl-3-glutaryl coA reductase (HMGR) genes, and specifically to the tomato *HMG2* (HMGR2) promoter element and its homologs from other plant species, to control the expression of protein and RNA products in plants and plant cells. The invention further relates to the use of sequences from the tomato *HMG2* promoter element or its homologs to modify or construct plant active promoters, which in turn may be used to control the expression of proteins and RNAs in plants and plant cells.

The tomato *HMG2* promoter element and its homologs have a variety of uses, including but not limited to

expressing or overexpressing heterologous genes in a variety of plant cell expression systems, plant cultures, or stably transformed higher plant species. Expression of the heterologous gene product may be induced by elicitor or chemical induction in plant cell expression systems, or in response to wounding, pathogen infection, pest infestation as well as elicitor or chemical induction in plants.

The use of the promoter elements described herein to engineer plants may have particular value. By way of illustration, and but by limitation, an agronomically important plant may be stably transformed with an *HMG2* or *HMG2* homolog promoter element or a promoter derived therefrom controlling a gene which confers resistance to pathogen infection or pest infestation. When such a plant is invaded by a pathogen or pest, the invasion will trigger the expression of the *HMG2* or *HMG2*-homolog promoter element-controlled resistance gene and cause the plant to become increasingly resistant to the pathogen or pest at the site of invasion.

The second aspect of the present invention relates to a method of producing gene products in harvested plant tissues and plant cell cultures. The method utilizes tissues of transgenic plants or transformed plant cells engineered with expression constructs comprising inducible promoters operably linked to the coding sequence of genes of interest. Tissues or cultures harvested from engineered plants or culture lines, respectively, may be induced to produce the encoded proteins or RNAs by treatments with the appropriate inducers or inducing conditions.

As demonstrated by the examples described herein, plants may be engineered with expression constructs comprising an inducible promoter, such as the *HMG2* promoter, operably linked to a sequence encoding a protein or RNA. Tissues harvested from the engineered plants may be induced to express the encoded protein or RNA by treatment with the appropriate inducer or inducing condition. With the post-harvest production method, the amount of encoded gene product produced

from an expression construct utilizing an inducible promoter may surpass that produced by an expression construct utilizing a strong constitutive promoter. Moreover, such harvested plant tissues can maintain, for up to two weeks after harvesting, their capability for strong induced production of the gene product encoded in the expression construct.

The post-harvest production method of the invention may be advantageously used to produce desired gene products that are adverse to plant or plant cell growth or development. In plants or plant cells engineered with expression constructs comprising inducible promoters operably linked to sequences encoding the desired gene product, the expression of the desired but plant-deleterious gene product would remain dormant during the growth of the plant or culture line, thus allowing for productive and efficient cultivation or culturing of the engineered plant or culture line. When the engineered plant or culture line has attained optimal condition for harvest or processing, the expression of the desired gene product may be artificially triggered by treatment with the appropriate inducer or inducing condition, which, in the case with expression constructs comprising the *HMG2* promoter, is mechanical wounding or elicitor or chemical induction. The desired product, either encoded by the induced gene or a compound produced by the induced gene product, may then be extracted from the induced plant, harvested plant tissue, or culture line after allowing for a short expression period.

The post-harvest production method of the invention also may be advantageously used to produce a labile gene product or a gene product that synthesizes a labile compound. In plants or plant cells engineered with expression constructs comprising inducible promoters operably linked to sequence encoding the desired gene product, the expression of the labile gene product or compound is deferred until the optimal condition is attained for harvesting of the plant or culture or processing of the desired product or compound. The synthesis of the desired product may then be induced in the plant or culture by treatment with the appropriate inducer or

inducing condition, and the desired gene product or synthesized compound expeditiously extracted from the induced plant or culture after allowing for a short expression period.

This aspect of the invention is based in part on the surprising findings that harvested plant tissues maintain significant capacity for expressing inducible genes after excision from the plant and that such capacity may actually increase with some storage time.

3.1. DEFINITIONS

The terms listed below, as used herein, will have the meaning indicated.

35S =	cauliflower mosaic virus promoter for the 35S transcript
CAT =	chloramphenicol acetyltransferase
cDNA =	complementary DNA
cis-regulatory element =	A promoter sequence 5' upstream of the TATA box that confers specific regulatory response to a promoter containing such an element. A promoter may contain one or more cis-regulatory elements, each responsible for a particular regulatory response.
DNA =	deoxyribonucleic acid
functional portion =	a functional portion of a promoter is any portion of a promoter that is capable of causing transcription of a linked gene sequence, e.g., a truncated promoter
gene fusion =	a gene construct comprising a promoter operably linked to a heterologous gene, wherein said promoter controls the transcription of the heterologous gene
gene product =	the RNA or protein encoded by a gene sequence
GUS =	1,3-β-Glucuronidase

heterologous gene =	In the context of gene constructs, a heterologous gene means that the gene is linked to a promoter that said gene is not naturally linked to. The heterologous gene may or may not be from the organism contributing said promoter. The heterologous gene may encode messenger RNA (mRNA), antisense RNA or ribozymes.
HMGR =	3-hydroxy-3-methylglutaryl CoA Reductase
HMG2 homolog =	A plant promoter sequence which selectively hybridizes to a known *HMG2* promoter (*e.g.*, the tomato *HMG2* promoter element disclosed herein), or the promoter of an HMGR gene that display the same regulatory responses as an *HMG2* promoter (*ie.* promoter activity is induced by wounding, pathogen-infection, pest-infestation, or elicitor treatment).
homologous promoter =	a promoter that selectively hybridize to the sequence of the reference promoter
mRNA =	messenger RNA
product of gene product =	a product produced by a gene product, e.g., a secondary metabolite
operably linked =	A linkage between a promoter and gene sequence such that the transcription of said gene sequence is controlled by said promoter.
RNA =	ribonucleic acid
RNase =	ribonuclease.

4. DESCRIPTION OF THE FIGURES

Figure 1. The mevalonate/isoprenoid pathway in plants. PGR, plant growth regulators.

Figure 2. Nucleic acid sequence comparison of tomato *HMG2* (SEQ ID NO:1) and tomato *HMG1* (SEQ ID NO:2). Sequences are aligned by the algorithm of Smith and Waterman (Devereux et al., 1984, Nucl. Acid Res. 12:387-395) which inserts gaps indicated by dots to optimize alignment. The transcriptional initiation sites are indicated by +1 and arrow. The translational start codons and TATAA boxes are underlined. The comparison ends at the first intron of each gene. Ambiguous bases are indicated by lower case letters.

Figure 3. Schematic of tomato *HMG2* promoter. **Panel A** The tomato *HMG2* genomic DNA insert of pTH295. **Panel B** The 2.5 kb EcoRI fragment of pTH295 used to general pDW101. pTH295 contains an approximately 7 kb HindIII fragment from the original lambda genomic clone inserted into the HindIII site of the multiple cloning region of bacterial plasmid pSP6/T7 (BRL). pDW101 contains a 2.5 kb EcoRI fragment of pTH295 inserted at the EcoRl site of plasmid bluescript SK-(Stratagene). Restriction endonucleases: Ac, AccI; Av, AvaI; Bg, BglII; E, EcoRI; Ev, EcoRV; H, HindIII; K, Kpnl, P, PstI; T, Taql; X, XbaI. The solid boxes represent the four exons encoding *HMG2* protein. The fragments below pDW101 indicate the location of the *HMG2* promoter sequences described in Figures 4, 5, and 6. The parentheses around *HMG2* Sequence 2 indicate that the exact location within this region has not yet been determined.

Figure 4. Nucleic acid sequence of *HMG2* Promoter Sequence I (SEQ ID NO:3). This 1388 bp sequence (SEQ ID NO:3) is from the 3'-end of the 2.5 kb EcoRI insert of pDW101. The transcriptional (+ 1) and translational (ATG) start sites are indicated. The TATAA box, PCR primer locations, and relevant restriction enzyme sites are underlined. Lower case letters indicate ambiguous bases.

Figure 5. Nucleic acid sequence of *HMG2* Promoter Sequence II (SEQ ID NO:4). The 480 bp sequence (SEQ ID NO:4)

is from an internal region of the 2.5 kb EcoRI insert of pDW101. This region spans approximately -1,039 to -2,000 base pairs upstream of the *HMG2* translation start site. The exact location of the sequence within this region remains to be determined. Two AT repeat motifs are underlined. An X indicates unknown bases; lower case letters are ambiguous bases.

Figure 6. Nucleic acid sequence of *HMG2* Promoter Sequence III (SEQ ID NO:5). The 415 bp sequence (SEQ ID NO:5) is from the 5'-end of the 2.5 kb EcoRI insert of pDW101. A 23 base palindromic sequence is underlined. Lower case letters are ambiguous bases.

Figure 7. Schematic representations of the promoter deletion clones, pDW201, pDW202 and pDW203, in pBI101. The open bars indicate the insert fragments from the tomato *HMG2* promoter. RB and LB are T-DNA border sequences. The 5' HindIII and 3' BamHI sites flanking each *HMG2* promoter region were generated within the sequence of the primers used to PCR-amplify these inserts.

Figure 8. Restriction map of plasmid pSLJ330.1. HMGA,EcoRI/BglII *HMG2* promoter fragment from pDW101; GUS, beta glucuronidase coding sequence; ocs 3', 3' region and polyadenylation site derived from *A. tumefaciens* octopine synthetase gene; p35S, promoter of the cauliflower mosaic virus 35S transcript; NPT, NPTII gene encoding neomycin phosphotransferase conferring kanamycin resistance; LB,RB, left and right border sequences of Ti plasmids required for transfer of DNA from *Agrobacterium* to plant cell.

Figure 9. Restriction map of plasmid pSLJ1911. This plasmid was used to transform plants with a 35S:GUS construct whose activity served as a benchmark to the various *HMG2*:GUS constructs. Abbreviations are as described in Figure 8.

Figure 10. Tissue specificity of *HMG2* Promoter expression in transgenic tobacco and tomato plants. Blue color indicates regions where GUS is active.

Pan 1 A. Anthers and pollen of *HMG2*:GUS compared to analogous tissues from 35S:GUS constructs (plants transformed with pSLJ330.1 or pSLJ1911, respectively).

Panel B. Trichomes of fully-expanded tobacco leaf (transformation vector pSLJ330.1).

Panel C. Root section of tomato seedling transformed with pSLJ330.1. Seedling was grown axenically on agar medium. Arrow indicates the location of lateral root initiation.

Figure 11. Defense-related *HMG2* Promoter expression in tobacco transformed with pSJL330.1.

Panel A. Leaf tissue 24 (2 left wells) and 48 hours after wounding and inoculating with water (W, mock) or soft-rotting bacterium *Erwinia carotovota* ssp. carotovora strain EC14 (EC).

Panel B. Tobacco seedlings (14 days post germination in sterile potting mix) 24 and 48 hours following inoculation at site indicated by arrow by placing *Rhizoctonia* infected oat seed in direct contact with stem.

Panel C. Leaf section of field grown tobacco showing GUS activity surrounding necrotic regions due to natural insect predation. Blue pigmentation at the outside edge of disk is due to wound response.

Figure 12. Nematode response of *HMG2*:GUS transgenic tomato (transformed with pSLJ330.1). Tomato seedlings were inoculated with *Meloidogyne hapla* second stage juveniles. At the indicated times after inoculation, roots were harvested, incubated overnight in X-Gluc to stain for GUS activity, and subsequently counter-stained with acid fucsin to visualize nematodes.

Panel A. Squash of root tip 2 days after inoculation.

Panel B. Root region 3 days post-inoculation. Arrow indicates region showing initial GUS activity.

Panels C, D, & E. Root tips showing characteristic nematode-induced swelling 7 days post-inoculation. Note in Panel E that uninfected root tips show no GUS activity.

Figure 13. Comparison of post-harvest expression of *HMG2* promoter-(hatched bars) and CaMV 35S promoter-(open bars) driven GUS gene in transgenic tobacco leaf tissue (transformed with pSLJ330.1 or pSLJ1911, respectively). Leaves were removed from plants at day 0, wound-induced by scoring and stored under moist conditions until harvest at the times indicated. GUS activity was determined as described in the text.

Figure 14: Post-harvest expression of an *HMG2*:GUS expression construct. The graph shows a comparison of the amount of GUS produced from freshly harvested leaves and that from harvested leaves after two weeks of 4°C storage. Leaves of mature field-grown plants containing the *HMG2*:GUS gene construct were harvested by excising the leaves at the petiole. The comparison was between the adjacent pairs of leaves from the same plant in order to ensure developmental similarity. One set of leaves, the "FRESH" samples, was processed shortly after harvest by wounding (i.e., by heavily scoring the leaf with a razor blade). The other set of leaves, the "WEEK 2" samples, was stored in "zip-lock" bags at 4°C for two weeks before wounding as described for the freshly harvested leaves. The GUS activity in the wounded leaves was determined immediately after wounding (the "Uninduced" sample) or after 48 hr of incubation at room temperature (the "48hr. Induced" sample). GUS activity is expressed as nM methyl-umbelliferone (MUG) released per min. per μg protein. The induced GUS activity from the stored leaf tissue was consistently equal to or greater than that from freshly harvested leaf tissue. The GUS activity of equivalent fresh leaf tissue of the untransformed parent cultivar is also shown (the "UNT" sample).

Figure 15: The effect of storage on the post-harvest expression of an *HMG2*:GUS expression construct. The graph shows a comparison of the amount of GUS produced from harvested leaves that have been stored for 2 or 6 weeks at 4°C or room temperature. Leaves of mature field-grown transgenic tobacco plants containing the *HMG2*:GUS gene construct were harvested by excising the leaves at the petiole. Adjacent leaves from each plant were sorted into treatment groups, i.e., storage in plastic bags at 4°C (the "4°C" samples) or storage at room temperature in dry burlap bags (the "Room Temp. Dry" samples). At the times indicated, a sample of the stored leaf tissue was processed by wounding and the GUS activity in the sample was determined at 0 and 48 hr. after wounding. GUS activity is expressed as nM methyl-umbelliferone (MUG) released per min. per μg protein. The induced GUS activity from the stored leaves, even after 6 weeks of storage, was equal to, or higher than, that of freshly harvested leaves.

5. DESCRIPTION OF THE INVENTION

One aspect of the present invention relates to the *HMG2* promoter and its homologs that are involved in the plant response to pathogen infections, pest infestations or mechanical wounding, and their use to drive the expression of heterologous genes.

The tomato *HMG2* promoter and its homologs control the expression of a 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) isozyme in plants. HMGR catalyzes the production of mevalonic acid, which is an intermediate in the biosyntheses of various secondary metabolites that have critical roles in plant defense responses and plant development. In most plant tissues, tomato *HMG2* promoter and its homologs remain dormant until their activity is triggered by pathogen infections, pest infestations or mechanical wounding. The induction of the *HMG2* and homologous promoters are mediated in part by so called "elicitor" compounds derived from constituents of plant (Darvill and Albersheim, 1984, Annu. Rev. Plant Physiol.

35:234) or plant pathogen cell wall or surface components. When induced, the *HMG2* promoter and its homologs effect rapid and strong expression of the genes under their control. The lack of significant constitutive expression and the rapidly inducible, strong expression characteristics of the *HMG2* and homologous promoters make them ideal elements for controlling the expression of various types of heterologous genes. Such genes include those encoding pathogen and pest resistance functions, products that are directly or indirectly deleterious to plant growth, labile products, or products involved in the biosyntheses of labile compounds, to name but a few.

According to this aspect of the invention, heterologous genes can be placed under the control of the tomato *HMG2* or homologous promoter elements or promoters derived therefrom, all described herein, and used to engineer cell culture expression systems, or transgenic plants. Expression of the heterologous gene will be induced in response to pathogen infection, pest infestation, mechanical wounding or chemical or elicitor treatment. For example, the induction of the heterologous gene in plant cell culture or plant may be induced by treatments with cell wall extracts of plant pathogens, purified elicitors contained within such extracts, or synthetic functional analogs of those elicitors. Alternatively, the expression of the heterologous gene in the plant may be induced by various bacterial or fungal plant pathogen infections. Similarly, the induction may also be triggered by infestation of the plant by pests such as insects and nematodes.

The second aspect of the invention relates to a method of producing gene products in plant tissues and cell cultures. The method utilizes the tissues of plants or cultures of plant cells that have been engineered with expression constructs comprising inducible promoters operably linked to sequences encoding the desired gene products. According to this aspect of the invention, the production of the desired gene product takes place in the plant tissue and

culture after they have been harvested and induced by treatment with the appropriate inducer or inducing condition.

The invention relates to the family of *HMG2* and *HMG2*-derived promoters and to the post-harvest production of gene products in plant tissues and cultures, and for the purpose of description only, the description of the invention will be divided into several stages: (a) inducible promoters that may be used in engineering the plant and plant cells for post-harvest production; (b) isolation, identification and characterization of such promoter sequences, including that of the *HMG2*; (c) identification and characterization of cis-regulatory elements within the inducible promoters, including those of the *HMG2* promoter, that can regulate other plant promoter sequences; (d) construction of expression vectors comprising heterologous genes of interest operably associated with an inducible promoters or derivatives thereof, including expression vectors comprising *HMG2* and derivative promoters; (e) engineering of expression vectors into plants or plant cells; (f) inducing the expression of said expression construct and the production of the gene product of interest in plant tissues or cell culture systems; and (g) the types of heterologous gene products that can be advantageously produced using the post-harvest production method, or more particularly under the control of the *HMG2* promoter elements and *HMG2*-derived promoters.

The various embodiments of the claimed invention presented herein are by the way of illustration and are not meant to limit the invention.

5.1. INDUCIBLE PROMOTERS

The inducible promoters that may be used in the expression vectors of the post-harvest production method of the invention can be any promoter whose activity is inducible in the host plant. Useful promoters include, but are limited to, those whose activities are induced by chemicals, biological elicitors, heat, cold, salt stress, light, wounding, desiccation, hormone, pathogen infection, or pest-

infestation. Table 3 lists some examples of inducible plant genes whose promoters may be used in the making of the instant invention.

Useful promoters are any natural or recombinant promoter whose expression can be induced in harvested plant tissue. Promoters with such a capability may be determined using any methods known in the art. For example, the promoter may be operably associated with an assayable marker gene such as GUS; the host plant can be engineered with the construct; and the ability and activity of the promoter to drive the expression of the marker gene in the harvested tissue under various conditions assayed.

TABLE 3

Inducible Plant Genes with Potential for Post-harvest Induction and Accumulation of Transgene Products. An asterisk designates those genes for which promoters have been isolated and characterized. References represent one to two representative references or relevant review article and are not intended to be exhaustive.

Genes/gene products	Functions	Sources of clones
Defense-Response Genes[a1]		
Phytoalexin biosynthesis		
Phenylpropanoid phytoalexin		
*Phenylalanine ammonia lyase (PAL)[2]	Enzyme, central pathway	Bean, parsley, potato, tomato
4-Coumarate CoA ligase (4CL)[3]	Enzyme, central pathway	Parsley, potato
*Chalcone synthase (CHS)[4,5]	Enzyme, Isoflavanoid branch	Bean, soybean, parsley
Chalcone isomerase (CHI)[6]	Enzyme, Isoflavanoid branch	Bean
Resveratrol (stilbene) synthase[7]	Enzyme, Isoflavanoid branch	Grapevine, peanut
Isoflavone reductase (IFR)[8]	Enzyme, Isoflavanoid branch	Alfalfa
Terpenoid phytoalexins		
*HMG-CoA reductase (HMG)[9,10]	Enzymes, central pathway	Tomato, tobacco, potato, rice
Casbene synthetase[11]	Casbene biosynthesis	Castor bean
Cell wall components		
Lignin		
*Phenylalanine ammonia lyase	See above	
Cinnamyl alcohol dehydrogenase (CAD)[12]	Lignin biosyn.	Tobacco
Caffeic acid o-methyltransferase[13]	Lignin biosyn.	Alfalfa, tobacco
Lignin-forming peroxidase[14]	Lignin polymerization	Tobacco, wheat
Hydroxyproline-rich glycoproteins (HRGP)[15,16]	Structural protein	Bean, tomato
Glycine-rich proteins (GRP)[15]	Structural protein	Bean, potato, pea, rice
Thionins[17]	Antifungal	Barley
Hydrolases, lytic enzymes		
*Chitinases (PR-P, PR-Q)[18-20]		
Class I chitinase, basic	Vacuolar, antifungal	Tobacco, bean, tomato
Class I and II chitinase, acidic	Extracellular, antifungal	Bean
Class II chitinase	Bifunctional lysozyme, chitinase	Cucumber, tobacco, barley, petunia
*β-1,3-Glucanase[21]	Antifungal, chitinase synergist	Bean, tobacco, potato, pea, rice, Arabidopsis
β-fructosidase[22]	Antifungal invertase	Tomato

TABLE 3 CONTINUED

Genes/gene products	Functions	Sources of clones
Others		
*Proteinase inhibitors (PI-I, PI-II)[23,24]	Trypsin-, chymotrypsin-inhibitors	Potato, tomato
Superoxide dismutase (SOD)[25]	Anti-oxidant enzyme	Tobacco, maize, tomato
Lipoxygenase[26]	Lipid peroxidation, jasmonate biosyn.	Arabidopsis
Additional "pathogenesis-related" prot.		
*PR1 family, PR2, PR3[27-29]	Unknown	Tobacco, bean, parsley, pea
Osmotin, PR5[30-32]	Antifungal, thaumatin-like	Tobacco, maize
Ubiquitin[33]	Protein degradation	Potato
Wound-Inducible Genes[a]		
*win1, *win2 (hevein-like)[34]	Chitin-binding prot.	Potato (hevein, rubber tree)
wun1, wun2[35]	Unknown	Potato
*nos, nopaline synthase[36]	Agrobacterium nutr.	Agrobacterium tumefaciens
ACC synthase[37]	Ethylene biosynthesis	Tomato, squash
HMG-CoA reductase hmg1[38]	Sterol/alkaloid synth.	Potato
3-deoxy--D-arabino-heptulosonate -7-phosphate synthase[39]	Lignin biosyn.	Potato, tomato
HSP70[33]	Heat-shock protein, chaparone	Potato
Salicylic acid inducible[40]		
acid peroxidase[14]	Lignin-forming	Tobacco
PR-proteins[40,41]	(see above)	Tobacco
Glycine-rich protein[41]	Cell wall protein	Tobacco
Methyl jasmonate inducible		
*vspB[42]	Vacuolar storage prot.	Soybean
Proteinase inhibitors I and II[43]	Trypsin, chymotrypsin inhib.	Potato, tomato
Heat-shock genes[43]		
HSP70[33]	Chaperonin	Potato
Ubiquitin	(see above)	
Cold-stress inducible[44]		
Drought, salt stress[45]		

TABLE 3 CONTINUED

Genes/gene products	Functions	Sources of clones
Osmotin[30-32]	Desiccation tolerance	Tobacco, maize
Hormone inducible		
Gibberellin		
α-amylase[46]	Starch degradation	Barley
Abscisic acid[45,47]		
EM-1, RAB, LEA genes[45]	Unknown, embryogenesis	Wheat, rice, maize, cotton
Ethylene		
Chitinase, phytoalexin biosyn. genes	(see above)	

a. Genes are transcriptionally activated in response to pathogens, defense elicitors, wounding and in some cases methyl jasmonate, salicylic acid, $HgCl_2$ or H_2O_2.

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47. Chandler and Robertson, 1994, Annu. Rev. Plant Physiol. Plant Mol. Biol. 45:113-142.

Useful promoters may be tissue-specific. Non-tissue-specific promoters (i.e., those that express in all tissues after induction), however, are preferred. More preferred are promoters that additionally have no or very low activity in the uninduced state. Most preferred are promoters that additionally have very high activity after induction.

In a particular embodiment, the inducible promoter is the *HMG2* promoter and derivatives thereof. The *HMG2* and derivative promoters, which in addition to having utility in the production method of the invention, also have utility in the *HMG2* promoter expression system of the invention. This aspect of the invention is also described herein in some detail.

5.2. PROMOTER ISOLATION AND CHARACTERIZATION

According to the present invention, functional portions of the promoters described herein refer to regions of the nucleic acid sequence which are capable of promoting transcription of an operably linked gene in response to an inducer or inducing condition at some point in the life cycle of the plant or cell culture, including after the tissue or culture has been harvested. In the case of the *HMG2* promoter, the functional portions are those regions which are capable of causing transcription of an operably linked gene in response to wounding, pathogen infection, pest infestation or chemical/elicitor treatment during the entire pattern of plant development.

Homologous nucleotide sequences is used herein to mean nucleic acid sequences which are capable of selectively hybridizing to each other. Selectively hybridizing is used herein to mean hybridization of DNA or RNA probes 50 bases or greater in length from one sequence to the "homologous" sequence under stringent conditions, e.g., washing in 0.1X SSC/0.1% SDS at 68°C for at least 1 hour (Ausubel, et al., Eds., 1989, Current Protocols in Molecular Biology, Vol. I, Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., New York, at page 2.10.3)

Nucleotide sequences homologous to the tomato *HMG2* promoter described herein refers to nucleic acid sequences which are capable of selectively hybridizing to the nucleic acid sequence contained in the approximately 2.2 kb EcoRI-Bql II fragment of pDW101 (NRRL Accession No. _____) in hybridization assays or which are homologous by sequence analysis (containing a span of 100 basepairs in which at least 75 percent of the nucleotides are identical to the sequences presented herein) and which has promoter activity identical or very similar to that of *HMG2*, i.e., wound, elicitor, pathogen, etc., inducibility. Such a promoter may be precisely mapped and its activity may be ascertained by methods known in the art, e.g., deletion analysis, expression of marker genes, RNase protection, and primer extension analysis of mRNAs preparations from plant tissues containing the homologous promoter operably linker to a marker gene.

Homologous nucleotide sequences refer to nucleotide sequences including, but not limited to, natural promoter elements in diverse plant species as well as genetically engineered derivatives of the promoter elements described herein.

Methods which could be used for the synthesis, isolation, molecular cloning, characterization and manipulation of the inducible promoter sequences, including that of the *HMG2* promoter, described herein are well known to those skilled in the art. See, e.g., the techniques described in Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd. Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989).

According to the present invention, the inducible promoter sequences, including that of the *HMG2* promoter, or portions thereof described herein may be obtained from an appropriate plant source, from cell lines or recombinant DNA constructs containing the inducible promoter sequences or genes sequences comprising the promoter sequences, and/or by chemical synthetic methods where the promoter sequence is known. For example, chemical synthetic methods which are well

known to those skilled in the art can be used to synthesize the *HMG2* sequences depicted in Figures 4, 5 and 6 herein (SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, respectively). The synthesized sequences can be cloned and expanded for use as promoter elements. Such synthetic sequences can also be used as hybridization probes to identify and isolate the desired promoter sequences from appropriate plant and cellular sources.

Alternatively, a desired promoter element may be identified and isolated by screening gene libraries. For example, a genomic DNA library may be screened for clones containing sequences homologous to known inducible genes, e.g., HMGR genes or, alternatively, inducible promoter sequences, e.g., HMGR promoters. For example, sequences from genomic or cDNA clones encoding the inducible gene or oligonucleotide probes corresponding to known amino acid sequences of the inducible protein may be used as hybridization probes to identify homologous clones in a genomic DNA library using standard methods. Such methods include, for example, the method set forth in Benton and Davis (Science 196:180 (1977)) for bacteriophage libraries, and Grunstein and Hogness (Proc. Nat. Acad. Sci. USA 72:3961-3965 (1975)) for plasmid libraries.

In instances where the species sources of the probe and the library are widely divergent, such as between a monocot and a dicot, the probe is preferably from a region encoding highly conserved amino acid residues of the inducible protein and the hybridization and wash are carried out at moderate stringencies, e.g., washing in 0.2X SSC/0.1% SDS at 42°C (Ausubel, et al., 1989, *supra*). Homologous gene sequences are those that are detected by the probe under such conditions and that encode a protein with 75% or greater amino acid homology with the protein encoded by the gene that is the source of the probe. In isolating *HMG2* homologous genes, useful hybridization conditions have been described in detail. See Park et al., 1992, Plant Mol. Biol. 20:327-331.

Clones containing homologous inducible gene sequences may be distinguished from other isogenes by their ability to detect, at high stringency hybridization conditions, mRNA transcripts that are induced by the expected inducing factor or condition. For example, *HMG2* homologous genes may be identified by the rapid appearance of hybridizing mRNA transcripts following wounding, pathogen infection, pest-infestation, or elicitor treatment (for an example of such an analysis see Yang et al., 1991, Plant Cell 3:397-405). The *HMG2* clones may also be identified by examining their ability to hybridize at moderate or high stringency condition to 5' untranslated region or 5' upstream region of known *HMG1* and *HMG2* (e.g., the 2.5 kb EcoRI fragment depicted in Figure 3) genes. *HMG2* clones can be identified as those that show a stronger signal with the *HMG2* probe than with the *HMG1* probe.

In other instances, where the species sources of the probe and the library are not widely divergent, such as between closely related plants, the probes used to screen such libraries may consist of restriction fragments or nucleotide sequences encoding the N-terminal 200 or so amino acids of the inducible protein, e.g., in the case of tomato *HMG2* HMGR see Park et al., 1992, Plant Mol. Biol. 20:327-331, portions thereof or nucleotide sequences homologous thereto. Retrieved clones may then be analyzed by restriction fragment mapping and sequencing techniques according to methods well known in the art.

In another approach, restriction fragments or nucleotide sequences of the inducible promoter or portions thereof and sequences homologous thereto may be used to screen genomic libraries to identify genomic clones containing homologous promoter sequences using the standard techniques described *supra*.

In an embodiment, the tomato *HMG2* promoter elements described in Figure 3, Figure 4, Figure 5, and Figure 6, portions thereof and sequences homologous thereto may be used to screen genomic libraries to identify genomic clones containing homologous promoter sequences. For example, the

2.5 kb EcoRI fragment containing the tomato *HMG2* promoter in plasmid pDW101 or portions thereof may be used to such ends.

In the above two approaches, the hybridization and wash conditions used to identify clones containing the homologous promoter sequences may be varied depending on the evolutionary relatedness between the species origins of the probe and the genomic library screened. The more distantly related organisms would require moderate hybridization and wash conditions. See *supra* for two wash conditions that can be used.

In yet another approach to the isolation of inducible promoter elements, known oligonucleotide sequences of inducible promoters can be used as primers in PCR (polymerase chain reactions) to generate the promoter sequences from any plant species. Similar to the hybridization approaches, the amplification protocol may be adjusted according to the evolutionary relatedness between the target organism and primer-source organism. The adjustments may include the use of degenerate primers and protocols well known in the art for amplifying homologous sequences. For a review of PCR techniques, see for example, Gelfind, 1989, PCR Technology. Principles and Applications for DNA Amplification, Ed., H.A. Erlich, Stockton Press, N.Y., and Current Protocol in Molecular Biology, Vol. 2, Ch. 15, Eds. Ausubel et al., John Wiley & Sons, 1988.

In instances where only internal sequences of an inducible promoter are known, sequences flanking such sequences may be obtained by inverse PCR amplification (Triglia et al., 1988, Nucl. Acids Res. 16:8186). The flanking sequences can then be linked to the internal promoter sequences using standard recombinant DNA methods in order to reconstruct a sequence encompassing the entirety of the inducible promoter.

The location of the inducible promoter within the sequences isolated as described above may be identified using any method known in the art. For example, the 3'-end of the promoter may be identified by locating the transcription

initiation site, which may be determined by methods such as RNase protection (Liang et al., 1989, J. Biol. Chem. 264:14486-14498), primer extension (Weissenborn and Larson, 1992, J. Biol. Chem. 267:6122-6131), reverse transcriptase/PCR. The location of the 3'-end of the promoter may be confirmed by sequencing and computer analysis, examining for the canonical AGGA or CAT and TATA boxes of promoters that are typically 50-60 base pairs (bp) and 25-35 bp 5'-upstream of the transcription initiation site. The 5'-end promoter may be defined by deleting sequences from the 5'-end of the promoter containing fragment, constructing a transcriptional or translational fusion of the resected fragment and a reporter gene, and examining the expression characteristics of the chimeric gene in transgenic plants. Reporter genes that may be used to such ends include, but are not limited to, GUS, CAT, luciferase, β-galactosidase and C1 and R gene controlling anthocyanin production.

According to the present invention, an inducible promoter element is one that confers to an operably linked gene in a plant or plant cell: 1) minimal expression in most plant tissues; and 2) induced expression following treatment by inducer or inducing condition known to trigger the expression of the promoter or gene from which the element is derived from.

In an embodiment, the *HMG2* promoter element is one that confers to an operably linked gene in a plant or plant cell: 1) minimal expression in most plant tissues; and 2) induced expression following wounding, pathogen infection, pest infestation or chemical/elicitor treatment. An *HMG2* promoter element may additionally confer specific developmental expression as in pollen, mature fruit tissues, and root tissues of the zone of lateral root initiation. See Table 1, which compares the expression characteristics of tomato *HMG2* promoter to the other known plant HMGR promoters.

According to the present invention, a promoter element comprise the region between -2,500 bp and +1 bp upstream of the transcription initiation site of inducible

gene, or portions of said region. In a particular embodiment, the *HMG2* promoter element comprises the region between positions -2,300 and +1 in the 5' upstream region of the tomato *HMG2* gene (see figure 3). Another embodiment of the *HMG2* promoter element comprises the region between positions -891 and +1 in the 5' upstream region of the tomato *HMG2* gene. An additional embodiment of *HMG2* promoter element comprises the region between positions -347 and +1 in the 5' upstream region of the tomato *HMG2* gene. Yet another embodiment of *HMG2* promoter element comprises the region between positions -58 and +1 in the 5' upstream region of the tomato *HMG2* gene. In further embodiments of the present invention, an *HMG2* promoter element may comprise sequences that commence at position +1 and continues 5' upstream up to and including the whole of the nucleotide sequence depicted in Figure 4, Figure 5 or Figure 6.

5.3. CIS-REGULATORY ELEMENTS OF PROMOTERS

According to the present invention, the cis-regulatory elements within an inducible promoter may be identified using any method known in the art. For example, the location of cis-regulatory elements within an inducible promoter may be identified using methods such as DNase or chemical footprinting (Meier et al., 1991, Plant Cell 3:309-315) or gel retardation (Weissenborn and Larson, 1992, J. Biol. Chem. 267-6122-6131; Beato, 1989, Cell 56:335-344; Johnson et al., 1989, Ann. Rev. Biochem. 58:799-839). Additionally, resectioning experiments may also be employed to define the location of the cis-regulatory elements. For example, an inducible promoter-containing fragment may be resected from either the 5' or 3'-end using restriction enzyme or exonuclease digests.

To determine the location of cis-regulatory elements within the sequence containing the inducible promoter, the 5'- or 3'-resected fragments, internal fragments to the inducible promoter containing sequence, or inducible promoter fragments containing sequences identified by footprinting or gel

retardation experiments may be fused to the 5'-end of a truncated plant promoter, and the activity of the chimeric promoter in transgenic plant examined as described in section 5.2. above. Useful truncated promoters to these ends comprise sequences starting at or about the transcription initiation site and extending to no more than 150 bp 5' upstream. These truncated promoters generally are inactive or are only minimally active. Examples of such truncated plant promoters may include, among others, a "minimal" CaMV 35S promoter whose 5' end terminates at position -46 bp with respect to the transcription initiation site (Skriver et al., Proc. Nat. Acad. Sci. USA 88:7266-7270); the truncated "-90 35S" promoter in the X-GUS-90 vector (Benfey and Chua, 1989, Science 244:174-181); a truncated "-101 nos" promoter derived from the nopaline synthase promoter (Aryan et al., 1991, Mol. Gen. Genet. 225:65-71); and the truncated maize Adh-1 promoter in pADcat 2 (Ellis et al., 1987, EMBO J. 6:11-16).

According to the present invention, a cis-regulatory element of an inducible promoter is a promoter sequence that can confer to a truncated promoter one or more of the inducible properties of the original inducible promoter. For example, an *HMG2* cis-regulatory element is an *HMG2* promoter sequence that can confer to a truncated promoter one or more of the following characteristics in expressing an operably linked gene in a plant or plant cell: 1) induced expression following wounding; 2) induced expression following pathogen infection; 3) induced expression following pest infestation; 4) induced expression following chemical elicitor treatment; or 5) expression in pollen, mature fruit, or other developmentally defined tissues identified as expressing *HMG2*. Further, an *HMG2* cis-regulatory element may confer, in addition to the above described characteristics, the ability to suppress the constitutive activity of a plant promoter.

5.4. INDUCIBLE PROMOTER-DRIVEN EXPRESSION VECTORS

The properties of the nucleic acid sequences are varied as are the genetic structures of various potential host

plant cells. The preferred embodiments of the present invention will describe a number of features which an artisan may recognize as not being absolutely essential, but clearly advantageous. These include methods of isolation, synthesis or construction of gene constructs, the manipulation of the gene constructs to be introduced into plant cells, certain features of the gene constructs, and certain features of the vectors associated with the gene constructs.

Further, the gene constructs of the present invention may be encoded on DNA or RNA molecules. According to the present invention, it is preferred that the desired, stable genotypic change of the target plant be effected through genomic integration of exogenously introduced nucleic acid construct(s), particularly recombinant DNA constructs. Nonetheless, according to the present inventions, such genotypic changes can also be effected by the introduction of episomes (DNA or RNA) that can replicate autonomously and that are somatically and germinally stable. Where the introduced nucleic acid constructs comprise RNA, plant transformation or gene expression from such constructs may proceed through a DNA intermediate produced by reverse transcription.

The present invention provides for use of recombinant DNA constructs which contain inducible promoter fragments, functional portions thereof, and nucleotide sequences homologous thereto. As used herein a functional portion of a promoter and a promoter homologous sequence are both capable of functioning as an inducible promoter. The functionality of such sequences can be readily established by any method known in the art. Such methods include, for example, constructing expression vectors with such sequences and determining whether they confer inducible expression to an operably linked gene. In particular embodiments, the invention provides for the use of tomato *HMG2* promoter fragments or sequences as depicted in Figures 3, 4, 5, and 6, functional portions thereof, and nucleotide sequences homologous thereto.

The inducible promoter elements of the invention may be used to direct the expression of any desired protein, or to direct the expression of an RNA product, including, but not limited to, an "antisense" RNA or ribozyme. Such recombinant constructs generally comprise a native inducible promoter or a recombinant inducible promoter derived therefrom, ligated to the nucleic acid sequence encoding a desired heterologous gene product.

A recombinant inducible promoter is used herein to refer to a promoter that comprises a functional portion of a native inducible promoter or a promoter that contains native promoter sequences that is modified by a regulatory element from a inducible promoter. For example, in particular embodiments, a recombinant inducible promoter derived from the *HMG2* promoter may comprise the approximately 0.17 kb, 0.46 kb or 1.0 kb HindIII-BamHI tomato *HMG2* promoter fragment of pDW201, pDW202 and pDW203, respectively (see Fig. 7). Alternatively, a recombinant inducible promoter derived from the *HMG2* promoter may be a chimeric promoter, comprising a full-length or truncated plant promoter modified by the attachment of one or more *HMG2* cis-regulatory elements.

The manner of chimeric promoter constructions may be any well known in the art. For examples of approaches that can be in such constructions, see section 5.3. above and Fluhr et al., 1986, Science 232:1106-1112; Ellis et al., 1987, EMBO J. 6:11-16; Strittmatter and Chua, 1987, Proc. Nat. Acad. Sci. USA 84:8986-8990; Poulsen and Chua, 1988, Mol. Gen. Genet. 214:16-23; Comai et al., 1991, Plant Molec. Biol. 15:373-381; Aryan et al., 1991, Mol. Gen. Genet. 225:65-71.

According to the present invention, where an inducible promoter or a recombinant inducible promoter is used to express a desired protein, the DNA construct is designed so that the protein coding sequence is ligated in phase with the translational initiation codon downstream of the promoter. Where the promoter fragment is missing 5'- leader sequences, a DNA fragment encoding both the protein and its 5' RNA leader sequence is ligated immediately downstream of the

transcription initiation site. Alternatively, an unrelated 5' RNA leader sequence may be used to bridge the promoter and the protein coding sequence. In such instances, the design should be such that the protein coding sequence is ligated in phase with the initiation codon present in the leader sequence, or ligated such that no initiation codon is interposed between the transcription initiation site and the first methionine codon of the protein.

Further, it may be desirable to include additional DNA sequences in the protein expression constructs. Examples of additional DNA sequences include, but are not limited to, those encoding: a 3' untranslated region; a transcription termination and polyadenylation signal; an intron; a signal peptide (which facilitates the secretion of the protein); or a transit peptide (which targets the protein to a particular cellular compartment such as the nucleus, chloroplast, mitochondria, or vacuole).

5.5. RECOMBINANT DNA CONSTRUCTS

The recombinant construct of the present invention may include a selectable marker for propagation of the construct. For example, a construct to be propagated in bacteria preferably contains an antibiotic resistance gene, such as one that confers resistance to kanamycin, tetracycline, streptomycin, or chloramphenicol. Suitable vectors for propagating the construct include plasmids, cosmids, bacteriophages or viruses, to name but a few.

In addition, the recombinant constructs may include plant-expressible, selectable, or screenable marker genes for isolating, identifying or tracking plant cells transformed by these constructs. Selectable markers include, but are not limited to, genes that confer antibiotic resistance, (e.g., resistance to kanamycin or hygromycin) or herbicide resistance (e.g., resistance to sulfonylurea, phosphinothricin, or glyphosate). Screenable markers include, but are not be limited to, genes encoding ß-glucuronidase (Jefferson, 1987, Plant Molec Biol. Rep 5:387-405), luciferase (Ow et al., 1986,

Science 234:856-859), B protein that regulates anthocyanin pigment production (Goff et al., 1990, EMBO J 9:2517-2522).

In embodiments of the present invention which utilize the *Agrobacterium tumefacien* system for transforming plants (see *infra*), the recombinant constructs may additionally comprise at least the right T-DNA border sequences flanking the DNA sequences to be transformed into the plant cell. Alternatively, the recombinant constructs may comprise the right and left T-DNA border sequences flanking the DNA sequence. The proper design and construction of such T-DNA based transformation vectors are well known to those skill in the art.

5.6. PRODUCTION OF TRANSGENIC PLANTS AND PLANT CELLS

According to the present invention, a desirable plant or plant cell may be obtained by transforming a plant cell with the nucleic acid constructs described herein. In some instances, it may be desirable to engineer a plant or plant cell with several different gene constructs. Such engineering may be accomplished by transforming a plant or plant cell with all of the desired gene constructs simultaneously. Alternatively, the engineering may be carried out sequentially. That is, transforming with one gene construct, obtaining the desired transformant after selection and screening, transforming the transformant with a second gene construct, and so on.

In an embodiment of the present invention, *Agrobacterium* is employed to introduce the gene constructs into plants. Such transformations preferably use binary *Agrobacterium* T-DNA vectors (Bevan, 1984, Nuc. Acid Res. 12:8711-8721), and the co-cultivation procedure (Horsch et al., 1985, Science 227:1229-1231). Generally, the *Agrobacterium* transformation system is used to engineer dicotyledonous plants (Bevan et al., 1982, Ann. Rev. Genet 16:357-384; Rogers et al., 1986, Methods Enzymol. 118:627-641). The *Agrobacterium* transformation system may also be used to transform as well as transfer DNA to monocotyledonous

plants and plant cells. (see Hernalsteen et al., 1984, EMBO J 3:3039-3041 ; Hooykass-Van Slogteren et al., 1984, Nature 311:763-764; Grimsley et al., 1987, Nature 325:1677-179; Boulton et al., 1989, Plant Mol. Biol. 12:31-40.; Gould et al., 1991, Plant Physiol. 95:426-434).

In other embodiments, various alternative methods for introducing recombinant nucleic acid constructs into plants and plant cells may also be utilized. These other methods are particularly useful where the target is a monocotyledonous plant or plant cell. Alternative gene transfer and transformation methods include, but are not limited to, protoplast transformation through calcium-, polyethylene glycol (PEG)- or electroporation-mediated uptake of naked DNA (see Paszkowski et al., 1984, EMBO J 3:2717-2722, Potrykus et al. 1985, Molec. Gen. Genet. 199:169-177; Fromm et al., 1985, Proc. Nat. Acad. Sci. USA 82:5824-5828; Shimamoto, 1989, Nature 338:274-276) and electroporation of plant tissues (D'Halluin et al., 1992, Plant Cell 4:1495-1505). Additional methods for plant cell transformation include microinjection, silicon carbide mediated DNA uptake (Kaeppler et al., 1990, Plant Cell Reporter 9:415-418), and microprojectile bombardment (see Klein et al., 1988, Proc. Nat. Acad. Sci. USA 85:4305-4309; Gordon-Kamm et al., 1990, Plant Cell 2:603-618).

According to the present invention, a wide variety of plants and plant cell systems may be engineered for the desired physiological and agronomic characteristics described herein using the nucleic acid constructs of the instant invention and the various transformation methods mentioned above. In preferred embodiments, target plants and plant cells for engineering include, but are not limited to, those of maize, wheat, rice, soybean, tomato, tobacco, carrots, peanut, potato, sugar beets, sunflower, yam, *Arabidopsis*, rape seed, and petunia.

5.7. SELECTION AND IDENTIFICATION OF TRANSFORMED PLANTS AND PLANT CELLS

According to the present invention, desired plants and plant cells may be obtained by engineering the gene constructs described herein into a variety of plant cell types, including but not limited to, protoplasts, tissue culture cells, tissue and organ explants, pollen, embryos as well as whole plants. In an embodiment of the present invention, the engineered plant material is selected or screened for transformants (*i.e.*, those that have incorporated or integrated the introduced gene construct(s)) following the approaches and methods described below. An isolated transformant may then be regenerated into a plant. Alternatively, the engineered plant material may be regenerated into a plant or plantlet before subjecting the derived plant or plantlet to selection or screening for the marker gene traits. Procedures for regenerating plants from plant cells, tissues or organs, either before or after selecting or screening for marker gene(s), are well known to those skilled in the art.

A transformed plant cell, callus, tissue or plant may be identified and isolated by selecting or screening the engineered plant material for traits encoded by the marker genes present on the transforming DNA. For instance, selection may be performed by growing the engineered plant material on media containing inhibitory amounts of the antibiotic or herbicide to which the transforming marker gene construct confers resistance. Further, transformed plants and plant cells may also be identified by screening for the activities of any visible marker genes (*e.g.*, the ß-glucuronidase, luciferase, B or C1 genes) that may be present on the recombinant nucleic acid constructs of the present invention. Such selection and screening methodologies are well known to those skilled in the art.

Physical and biochemical methods also may be used to identify a plant or plant cell transformant containing the gene constructs of the present invention. These methods

include but are not limited to: 1) Southern analysis or PCR amplification for detecting and determining the structure of the recombinant DNA insert; 2) northern blot, S-1 RNase protection, primer-extension or reverse transcriptase-PCR amplification for detecting and examining RNA transcripts of the gene constructs; 3) enzymatic assays for detecting enzyme or ribozyme activity, where such gene products are encoded by the gene construct; 4) protein gel electrophoresis, western blot techniques, immunoprecipitation, or enzyme-linked immunoassays, where the gene construct products are proteins; 5) biochemical measurements of compounds produced as a consequence of the expression of the introduced gene constructs. Additional techniques, such as *in situ* hybridization, enzyme staining, and immunostaining, also may be used to detect the presence or expression of the recombinant construct in specific plant organs and tissues. The methods for doing all these assays are well known to those skilled in the arts.

5.8. EXPRESSION OF HETEROLOGOUS GENE PRODUCTS IN TRANSGENIC PLANTS

The present invention may be advantageously used to direct the expression of a variety of gene products. These gene products include, but are not limited to, proteins, anti-sense RNA and ribozymes.

In embodiments of the present invention, a inducible promoter or a recombinant inducible promoter may be used, in post-harvest production, to express in plants and plant cell cultures a variety of high valued protein products, including, but not limited to, pharmaceutical and therapeutic enzymes and proteins. Such protein products may include, for example, various peptide-hormones, cytokines, growth factors, antibodies, blood proteins and vaccines. Further, these promoter elements may also be used to express in plants and cell cultures multiple enzymes of complex biosynthetic pathways, the induction of which would confer to host plant or plant cell the ability to produce complex biochemicals and

biologicals. Examples of such products include secondary metabolites such as alkaloids, antibiotics, pigments, steroids and complex biological structures such as intact or defective viruses or viral particles. Additionally, these promoter elements may also be used to express various types of lytic and processing enzymes that can convert what would be otherwise unusable or low quality plant compounds or constituents into useful or high quality compounds or chemical feedstocks. Examples of such products include cellulases, lignases, amylases, proteases, pectinases, phytases, etc. Furthermore, a inducible promoter or a recombinant inducible promoter may also be used to express RNA products such as antisense RNA and ribozymes. In particular embodiments, *HMG2* or *HMG2*-derived promoter elements which confer wound-inducible and/or elicitor-inducible gene expression are used to express the above-mentioned products.

In the above embodiments, the inducible promoter or recombinant inducible promoter, including the wound- and/or elicitor-specific *HMG2* and *HMG2*-derived promoters, may be advantageously used to direct the "post-harvest" production and accumulation of the desired direct or indirect gene products. That is, the production of the desired products does not occur during normal growth of the plant or the cell culture, but only occurs after the plant or the cell culture (e.g., callus culture) is mechanically macerated and/or elicitor-treated shortly before, during, or shortly after, harvesting the plant or cell cultures. (For a general reference describing plant cell culture techniques that could be used in conjunction with the "post-harvest" production approach disclosed here, see Handbook of Plant Cell Culture, Vol. 4, Techniques and Applications, etc., Evans, D.A., Sharp, W.R. and Ammirato, P.V., 1986 Macmillan Publ., New York, New York).

Any plant tissue or culture lines of plants or plant cells engineered with the expression construct described herein may used in the production of desired direct or indirect gene products. Useful plant tissue (and organs)

include, but are not limited to, leaf, stem, root, flower, fruit and seed.

Where the production of the desired indirect gene product, e.g., a secondary metabolite, requires two or more gene functions, it may be advantageous to engineer the host plant or plant cell with several expression constructs, wherein each construct comprises the same inducible promoter controlling the expression of each required gene function, so as to enable the coordinate expression of all required gene functions.

The induction of the harvested tissues and cultures from the engineered plants and cells may be by any means known in the art for the particular inducible promoter used in the expression construct. For example, to induce expression from an expression construct vector comprising an *HMG2* or *HMG2*-derived promoter, the harvested tissue or culture may be physically wounded by maceration, treated with biological elicitors, infected with an appropriate pathogen, etc.

The induction of the harvested plant tissue or culture may be done immediately after harvesting or the harvested tissue or culture may be stored and then subsequently induced. The storage of the harvested plant tissue or culture may be by any procedure or method known in the art that optimally preserves the gene expression capability of the stored material.

In particular embodiments, harvested leaves can be stored at 4°C in sealed containers or at room temperature in air permeable containers for up to 6 weeks before inducing the expression of the desired gene products. Preferably, the stored leaves are induced at about two weeks after harvest.

The induced tissue or culture may be incubated at room temperature for up to a week to allow for the expression of the induced gene(s) and accumulation of the desired direct or indirect gene product before the tissue or culture is processed for isolating the desired direct or indirect gene product. In particular embodiments, induced leaf tissue is

incubated at room temperature for approximately 48 hr. before the leaf tissue is processed.

The application of inducible promoter or recombinant inducible promoters to produce the above mentioned types of products is particularly significant given that many such products may be either labile or deleterious to cellular metabolism or plant growth. Thus, their efficient and optimal production, in many instances, may be best achieved by the use of inducible, particularly wound-inducible or elicitor-inducible, promoters coupled with a post-harvest induction protocol. As explained previously, harvesting of plants or plant parts for later use does not normally kill or harm plant tissues if they are maintained in an environmentally suitable condition.

In another embodiment, an *HMG2* or *HMG2*-derived promoter which confers pathogen-induced expression may be used to express a variety of disease resistance genes during a pathogen infection. Examples of such resistance genes include virus coat protein, anti-sense RNA or ribozyme genes for anti-virus protection (Gadani et al., 1990, Arch. Virol 115:1-21); lysozymes, cecropins, maganins, or thionins for anti-bacterial protection; or the pathogenesis-related (PR) proteins such as glucanases and chitinases for anti-fungal protection.

In a further embodiment, an *HMG2* or *HMG2*-derived promoter which confers pest infestation-induced expression may be used to express a variety of pest resistance genes during an insect or nematode infestation. Examples of useful gene products for controlling nematodes or insects include *Bacillus thuringiensis* endotoxins, protease inhibitors, collagenases, chitinase, glucanases, lectins, glycosidases, and neurotoxins.

The *HMG2* and *HMG2*-derived promoters have a number of characteristics that make them particularly useful for expressing pathogen and pest resistance genes. These characteristics include these promoters' very low background activity in most uninduced tissues of the plant, their rapid induction and strong activity once induced and the relatively site-specific nature of the induced response. These combined

characteristics make the *HMG2*-controlled resistance functions highly efficient by limiting the resistance response to the time and place that such plant responses would be the most effective (*i.e.*, the site of the pathogen or pest ingress).

In yet another embodiment, an *HMG2* or *HMG2*-derived promoter which confers pollen-specific expression may be used to engineer male-sterile plants. A pollen-specific *HMG2* or *HMG2*-derived promoter may be used to express gene functions that interfere with vital cellular processes such as gene expression, cell division or metabolism. Examples of such functions include RNases, DNases, anti-sense RNAs and ribozymes. The use of pollen-specific *HMG2* or *HMG2*-derived promoters would limit the expression of such deleterious function to pollen tissue without affecting other aspects of normal plant growth development.

6.0. EXAMPLE: ISOLATION AND CHARACTERIZATION OF THE TOMATO *HMG2* PROMOTER AND *HMG2* GENE FUSIONS

The isolation of the tomato *HMG2* promoter is described here. The disclosed approach is generally applicable to the isolation of homologs of any cloned promoter, and is particularly applicable to the isolation of homologs of the tomato *HMG2* promoter, from other plant species. The approach uses a gene sequence containing the coding region of an HMGR to screen a genomic library under low stringency hybridization conditions. In the present example, the probe used was a fragment containing a region of the yeast HMGR that shows a high degree of amino acid sequence conservation with the hamster HMGR. Positive clones are isolated and subcloned where necessary, and the hybridizing region is sequenced along with the flanking sequences. Sequences containing the *HMG2* gene are identified based on their nucleotide sequence comparisons with known HMGR genes and their divergence from known *HMG1* genes (see Park et al., 1992, Plant Mol. Biol. 20:327-331).

Promoter:reporter gene fusions are used here to localize the *HMG2* promoter element within the cloned sequence 5' upstream of the HMGR coding sequence. One goal of the analyses is to demonstrate the tissue-specificity and defense-related and post-harvest inducibility that the 2.3 kb *HMG2* promoter confers on operably linked heterologous genes. The second goal of the analysis is to determine in transgenic plants the smallest 5' upstream fragment that would confer the complete array of regulatory responses of the *HMG2* gene. Thus, commencing with a 2.5 kilo-base pair (kb) fragment 5' upstream of the *HMG2* transcription initiation site, smaller promoter element fragments are generated using PCR leaving out incrementally larger tracts of sequences from the 5'-end of the, 2.5 kb, fragment. Gene fusions are constructed by ligating the 2.3 kb EcoRI/BglII fragment and each of the smaller, PCR-generated fragments to β-glucuronidase (GUS) reporter genes. The activities of the fusion genes are examined in transgenic plants to determine the location of the *HMG2* promoter.

6.1. MATERIALS AND METHODS

6.1.1 PLANT AND FUNGAL MATERIAL

Tomato (*Lycopersicon esculentum* cvs. Gardener and Vendor) and tobacco (*Nicotiana tobacum* cvs. Xanthi and NC95) plants were grown under greenhouse conditions. Seedlings for transformation experiments were grown axenically as described below. For elicitor treatments of suspension cultured plant cells, tomato EP7 cells were maintained in the dark in a modified MS medium. *Verticillium alboatrum* (race 1) and *Fusarium oxysporum* (race 1), provided by Dr. Martha Mutschler (Cornell University, Ithaca, NY), were maintained on 2.4% potato dextrose agar and grown in 2.4% liquid medium for cell wall isolation. Fungal elicitor, the high molecular weight material heat-released from isolated mycelial walls was obtained and measured as described (Ayers et al., 1976, Plant Physiol. 57:760-765). *Rhizoctonia solani* strains RS51 and

R992 were provided by Dr. Charles Hagedorn (Virginia Polytechnic Institute and State University).

6.1.2. GENOMIC LIBRARY SCREENING

Recombinant clones (500,000) of a tomato genomic DNA library (*L. esculentum* cv. VFNT Cherry) constructed in lambda Charon 35 were screened by plaque hybridization. Hybridization probe was the 1.75 kb EcoRI fragment of pJR326, (provided by Dr. Jasper Rine of University of California, Berkeley, CA) which contains the region of *S. cerevisiae HMG1* most highly conserved with hamster HMGR (Basson et al., 1986, Proc. Natl. Acad. Sci. USA 83:5563-5567). Initial screening was at low stringency conditions (*e.g.*, 30% formamide, 6X SSC, 5X Denhardt solution, 0.1% SDS, 100 ug/ml salmon sperm DNA at 37°C for 24 hours; final wash conditions were 0.2X SSC at room temperature). Plaques giving positive hybridization signals were carried through at least three rounds of purification prior to further characterization. A 7 kb HindIII fragment of one clone (designated TH29) was subcloned into the HindIII site of transcription vector pSP6/T7 (Bethesda Research Laboratories (BRL), Gaithersburg, MD) and designated pTH295 (Figure 3).

6.1.3. NUCLEIC ACID ISOLATION

Total DNA was isolated from tomato leaves according to the method of Draper and Scott, (Plant Genetic Transformation and Gene Expression, 1988, Eds. Draper et al., Blackwell Scientific, Palo Alto, CA, pp211-214)). For RNA isolation, suspension cultured tomato cells treated with elicitors, healthy roots, stems, and leaves treated by wounding (cut into 1 mm slices with a razor blade and compressed with a pestle), or intact fruit at various stages of development were stored at -70°C. Total RNA was isolated from 1 to 3 g fresh weight of tissue ground in liquid nitrogen and homogenized directly in a phenol:0.1 M Tris (pH 9.0) emulsion as described previously (Haffner et al., 1978, Can. J. Biochem. 56:7229-7233).

6.1.4. HYBRIDIZATION ANALYSIS

For genomic Southern analyses, 10 μg/lane total DNA was digested with restriction endonucleases, separated on 0.8% agarose gels, and transferred to Nytran membranes using conditions recommended by manufacturer (Schleicher and Schuell, Keene, NH). For Northern analyses, total RNA (5 to 20 μg/lane) was denatured by treatment with glyoxal prior to electrophoresis in 1.2% agarose for gel analyses or application directly to Nytran filters utilizing a slot blotting apparatus. For hybridizations aimed at revealing all members of the HMGR gene family, probes derived from the 3' end of the gene which is most highly conserved between species (Basson et al., 1988, Mol. Cell. Biol. 8:3797-3808) were used. Either the 1.5 kb EcoRI fragment of pTH295 (Figure 3; Yang et al., 1991, Plant Cell 5:397-405) or a 486 bp *HMG2* cDNA clone from this region derived from pCD1 (NRRL Accession No. ____) were 32P-labeled by random-primer methods (Multi-prime Labeling System, Amersham, U.K.). Membranes were prehybridized overnight without labeled probe and hybridized in the presence of 32P-labeled probe for 24 to 48 hr at 42°C in solution containing 40% formamide, 6X SSC, 5X Denhardt solution, 5 mM EDTA, 0.1% SDS, 100 μg/ml salmon sperm DNA (Sigma). Final wash conditions were 0.1X SSC, 0.1% SDS, 1 hr at room temperature. For analyses aimed at monitoring hybridization of sequences specific only for the *HMG2* isogene encoded by pTH295, the 0.7 kb AvaI-EcoRI fragment encoding 5-untranslated regions and the 5' end of the gene or a smaller 340 bp subclone derived from this fragment and lacking the upstream region, was utilized. Hybridization conditions were as described above except that 50% formamide and 5X SSC were used. Following hybridization, membranes were washed (final wash used 0.1X SSC at 50°C for 1 hr) to remove unbound label prior to X-ray film exposure.

6.1.5. *HMG2* PROMOTER:REPORTER GENE FUSIONS

The 2.5 kb EcoRI fragment of the *HMG2*-containing clone pTH295 was inserted into the EcoRI site of a Bluescript

SK-vector (Stratagene) and designated pDW101 (Figure 3; NRRL Accession No. ____). Digestion of this vector with EcoRI and BglII releases a fragment (about 2.3 kb) which contains sequences comprising sequences encoding the first five N-terminal amino acids of the *HMG2* HMGR and extending approximately 2.3 kb 5' upstream of these coding sequences. This fragment was ligated to the GUS gene at an NcoI site to create an in-frame fusion with the ATG start codon of GUS and inserted into a modified pRK290 plasmid to generate pSLJ330.1 (Figure 8). This plasmid was constructed at the Sainsbury Laboratory, John Innes Institute (Norwich, U.K.) in collaboration with Jonathan Jones using transformation vectors provided by Dr. Jones. Subsequent *HMG2* promoter constructs were inserted into plant transformation/expression vectors of the pBI series (ClonTech). The pSLJ and pBI plasmids containing the fusion genes were introduced in Agrobacterium tumefaciens strain LBA4404 (Clontech) by tri-parental matings using the helper plasmid pRK2013.

6.1.6. SEQUENCING OF *HMG2* PROMOTER DELETIONS

Progressively larger deletions from the 3' and 5' ends of the 2.5 kb promoter region of *HMG2* contained in pDW101 were generated utilizing the Exonuclease III/Mung Bean Nuclease reaction kit purchased from Stratagene (La Jolla, CA). Double-stranded pDW101 was digested with *Bam*HI and *Sac*I (both of which cut in the vector portion of the plasmid), resulting in a linear piece of DNA with a 5' overhang and a 3' overhang. After phenol:$CHCl_3$ extraction to remove the restriction enzymes, the DNA was precipitated with 100% EtOH and dried. The digested DNA was then treated with 20 units Exonuclease III/μg DNA to create a segment of single-stranded DNA. Aliquots of the reaction were removed every 45 seconds and added to tubes which contained Mung Bean Nuclease buffer. Mung Bean Nuclease was then used to digest the single-stranded portion of the DNA. This process was calculated to remove approximately 300 bp for every time point. The enzymes were extracted by treatment with lithium chloride and the DNA

precipitated by the addition of sodium acetate and ethanol. This process was followed by a fill-in reaction using the Klenow fragment to ensure the presence of blunt ends for subsequent ligation. The resulting plasmids were transformed into *Escherichia coli*. Double-stranded DNA sequencing was performed using Sequenase 2.0 (United States Biochemical, Cleveland, OH) according to protocols provided by the manufacturer. Electrophoresis was carried out on 5% HydroLink Long Ranger acrylamide gels (AT Biochem, Malvern, PA).

6.1.7. GUS GENE FUSIONS WITH *HMG2* PROMOTER DELETIONS

Deletions in the *HMG2* promoter were created using PCR methodology. The primers used (designated in Figure 4), and the approximate size fragments obtained were: primers #22 and #18, 1000 bp; primers #20 and #18, 460 bp; primers #19 and #18, 170 bp. Primers #19, #20 and #21 generated a flanking HindIII site; primer #18 generated a flanking BamH1 site. The PCR reactions contained 1.5 mM $MgCl_2$, 200 μM each dNTP, 2.5 units Taq polymerase, 1X Taq polymerase buffer, 100 ng pDW101, and approximately 40 pmol each primer. Three cycles were used: Cycle 1 (1x): 95°C-5 min, 72°C-5 min, 58°C-2 min, 72°C-15 min, with the addition of the Taq polymerase after the first 72°C incubation; Cycle 2 (40x): 95°C-1 min, 58°C-2 min, 72°C-3 min; Cycle 3 (1x): 72°C-15 min. The DNA fragments generated were electrophoresed on a 1.4% agarose gel to verify the sizes. Polyacrylamide gel-purified fragments were digested with the restriction enzymes *Hin*dIII and *Bam*HI. These fragments were then ligated into the binary vector pBI101 which had been similarly digested (Figure 7). The resulting plasmids, pDW201 (NRRL Accession No. ____), pDW202 (NRRL Accession No. ____), and pDW203 (NRRL Accession No. ____) were transformed into *E. coli* strain DH5α and their sequences verified. The binary plasmids were introduced into *Agrobacterium* strain LBA4404 using tri-parental mating.

6.1.8. TRANSFORMATION OF GENE CONSTRUCTS INTO PLANTS

The *HMG2* promoter:reporter gene fusions were transformed into tobacco according to the leaf disk transformation protocol of Horsch et al. (Horsch et al., 1985, Science, 227:1229). Prior to co-cultivation with appropriately engineered *Agrobacterium tumefaciens*, leaf disks excised from axenically grown tobacco seedlings were incubated for 8 hours on sterile filter paper overlaying a lawn of cultured tobacco "nurse" cells spread on a feeder plate (modified MS medium containing Nitsch vitamins, 100mg/L myo-inositol, 30 gm/L sucrose, 1 mg/L 2,4-D, 0.4mg/L BAP, 8 gm/L agar). Co-cultivation was accomplished by submersing the leaf disks in a suspension of *A. tumefaciens* at a concentration of 1 x 10^9 cells/ml, followed by vacuum infiltration (3 x 1 min). The leaf disks were then returned to the nurse plates and incubated for 48 hours at 25°C with indirect light. Disks were then transferred to selection/regeneration plates (MS salts, Nitsch vitamins, 100/L mg myo-inositol, 20 gm/L sucrose, 2 mg/L zeatin, 4 gm agar/L) which contain carbenicillin (500 μg/ml) and an appropriate antibiotic for transformation selection. Plates were returned to the growth chamber (25°C, 18 hr light). Resulting shoots were excised and transferred to rooting media (MS salts, Nitsch vitamins, 100 mg/L myo-inositol, 30 g/L sucrose, 4 gm/L agar, and IAA and kinetin at final concentrations of 10 μM and 1 μM, respectively). Rooted plantlets were then transferred to soil and moved to the greenhouse.

Transformation of tomato was performed in a similar manner with the exception of the source of tissue. Cotyledons cut from aseptically grown tomato seedlings were cut under water using a sterile scalpel into 0.5-1.0 cm explants. Explants were then incubated on feeder plates for 8 hours prior to transformation.

6.1.9. EXAMINATION OF GUS EXPRESSION IN TRANSGENIC PLANTS

Histochemical analysis of GUS activity in transgenic plant tissues was carried out using standard techniques well known in the art. For routine analyses, plant organs or tissue sections were submerged in a solution of 1 mM X-gluc in 50 mM phosphate buffer (pH 7) and 0.1% Triton X-100. Infiltration of the substrate was facilitated by 3 x 1 minute vacuum infiltration. The amount of vacuum infiltration was varied based on the thickness and hardiness of the tissue to be tested. Tissue was incubated 4-12 hours at 37°C. Following development of GUS staining, leaf tissue was generally treated with ethanol (boil 2 minutes in 95% or incubate overnight in 95%) to remove chlorophyll. GUS activity was also monitored in cell-free extracts using the fluorescent substrate MUG by standard protocol (Jefferson, 1987, Plant Mol. Biol. Rep. 5:387-405). GUS activity was expressed as nmol MU/min/μg protein where protein was determined by the method of Bradford (Pierce Coomassie Plus Protein Assay Reagent) using BSA as the standard.

6.1.10 BACTERIAL INFECTION INDUCTION OF *HMG2* PROMOTER ACTIVITY

A 5 ml culture of *Erwinia carotovora* ssp. *carotovora*, strain EC14, causal agent of soft rot, was grown overnight from a single colony in LB medium at 25°C. The culture was centrifuged and resuspended in 1 ml distilled water (OD_{600} = 3.54-3.82). Leaves, 6-8 inches in length, of greenhouse grown transgenic tobacco carrying the *HMG2*:GUS construct were excised at the petiole and placed on water-moistened filter paper in a large petri dish. The tip of a micropipettor was used to gently wound the top surface of the leaf while depositing 2 μl of distilled water (mock inoculation) or EC14 suspension. The plates were closed and placed in a plastic container with a tightly-fitting lid (e.g., Tupperware) that had been lined with water-saturated paper towels and incubated at 28°C in the dark. Samples were

harvested at 24 hour intervals by excising a leaf section surrounding the site of inoculation using a hole punch or cork-borer and processed for histochemical analysis as described above.

6.1.11 FUNGAL INFECTION INDUCTION OF *HMG2* PROMOTER ACTIVITY

Seeds of transgenic tobacco were surface sterilized by soaking in 30% bleach, rinsed 4 times with sterile water and germinated in autoclaved potting mix (ProMix BX; Premier Brands, Inc, Stamford, CT) in 4 x 4 inch PlantCons (ICN Biomedicals, Inc, Irvine, CA). Fungal inoculations were done by placing a fungal-infested oat seed in contact with the seedling hypocotyl. *Rhizoctonia solani* strains RS51 and R992 were inoculated as agar plugs into moist, sterilized oat seed and grown for 8 days at room temperature prior to inoculation on plants. At 24 hour intervals, seedlings were removed with care was taken to limit contact to leaves to minimize wounding of the seeding near the site of inoculation, and the soil removed by dipping the roots into water. The entire seedlings, 1-2 inches in length, were then processed for histochemical GUS determination as described above. Tomato seedlings grown on agar (1/10 x MS salts plus 1% agar) were also inoculated with a small agar plug of plate-grown *Rhizoctonia solani*. This method of inoculation was less effective than the soil-grown seedling method because the agar medium supported vigorous fungal growth.

6.1.12 NEMATODE INFESTATION INDUCTION OF *HMG2* PROMOTER ACTIVITY

T1 generation tomato seedlings were grown for 10 days on seed germination media (1/10 x MS salts, and 10 mg/L myo-inositol, 3 gm/L sucrose, 6 gm/L agarose) in 4 x 4 inch Plantcons. Approximately 2000 nematodes (either *Melodigyne incognita* or *M. hapla*) were applied to the top of the media; Plantcons were then placed in a 25°C growth chamber. Seedlings were harvested at 1, 2, 3, 5, and 7 days post-

inoculation by slowly pulling seedlings from the agarose, gently removing agarose from roots, and cleanly cutting top of seedling. Roots were analyzed for GUS expression histochemically.

6.1.13 POST-HARVEST WOUND INDUCTION OF *HMG2* PROMOTER ACTIVITY

Leaves approximately 8 inches in length were removed from *HMG2*:GUS and 35S:GUS tobacco plants and were wounded by heavily scoring with a razor blade. Sections of each leaf were immediately removed, weighed, then frozen in liquid nitrogen and stored at -70°C. Additional sections of leaf were removed, weighed, and frozen at 24 and 48 hours post-harvest. Leaf extract was obtained by grinding in MUG extraction buffer (Jefferson, 1987, Plant Mol. Biol. Rep. 5:387-405) with a mortar and pestle. Protein concentrations were determined by the Bradford method. MUG assays were performed according to the method of Jefferson (Jefferson, 1987, id.); activity was expressed as nmol MU/min/μg protein.

6.1.14 FIELD PERFORMANCE OF TRANSGENIC PLANTS CONTAINING *HMG2*:GUS GENE FUSIONS

Seeds of two independent transformants of each of two cultivars (Xanthi and NC-95) showing high levels of wound-inducible *HMG2*:GUS activity were used for initial field tests. Seedlings of transgenic tobacco carrying a 35S:GUS construct (SJL1911, Figure 9) were also planted to use as controls. Transgenic seedlings were grown in the greenhouse for approximately 4 weeks prior to transfer to the field. Test I involved 300 plants (seven genotypes) planted (automated planter) at the Virginia Tech Southern Piedment Agricultural Experiment Station in Blackstone, Virginia. Test II involved 175 plants (7 genotypes) planted as a randomized block in experimental plots at Virginia Tech, Blacksburg, Virginia. Leaf material is harvested at various times during the growing season (including just prior to and 2 weeks following routine topping of the plants) to determine field levels of basal *HMG2*

promoter activity and post-harvest induction of transgene activity. *HMG2*:GUS expression in response to natural pathogen pressure (including, but not limited to, cyst nematodes, aphids, beetle and hornworm predation, tobacco mosaic virus) is monitored by histochemical analysis.

6.2 RESULTS

6.2.1 *HMG2* PROMOTER ACTIVITY

In order to delineate regulation of *HMG2* expression, 2.3 kb of *HMG2* upstream sequences were fused to the GUS reporter gene in plasmid pSJL330.1 and used for *Agrobacterium tumefaciens*-mediated plant transformation. More than 20 independent GUS-expressing tobacco transformants containing 1-4 copies of this construct or a 35S:GUS control construct generated by transformation with plasmid pSJL1911 (Figure 9; provided by Dr. Jonathon Jones, John Innes Institute, Norwich, U.K.) were generated. The 35S promoter is a high-level constitutive plant promoter derived from the cauliflower mosaic virus, (Benfey et al., 1989, EMBO J. 8:2195-2202; Fang et al., 1989, Plant Cell 1:141-150).

6.2.2 TISSUE SPECIFIC ACTIVITY OF THE *HMG2* PROMOTER

Plants expressing the *HMG2*:GUS constructs provide a powerful tool for assessing tissue-specificity of *HMG2* expression. Histochemical analyses of GUS activity indicated that *HMG2* is expressed in unstressed plants in the hypocotyl region of seedlings (region of shoot which breaks through soil), in trichomes (plant hairs on leaf surface important in insect, pathogen-resistance, Gershenzon et al., 1992, Anal. Biochem. 200:130-138), and in pollen (Figure 10). Expression in these tissues could be defense-related. Previous reports have shown that some defense-related genes are elevated in pollen (Kononowicz, 1992, Plant Cell 4:513-524). However, significant levels of GUS activity in the primary root of young tomato seedlings were also observed at sites of lateral root initiation. The significance of this expression is

currently unknown. It does not appear to be related to cell division because no GUS expression is found in the zone of cell division in the root tip. The activity of the *HMG2*:GUS constructs has also been determined in ripening fruit of transgenic tomato plants. Fruit at various stages of development were harvest and tested for GUS activity in cell-free extracts or analyzed for tissue specificity using histochemical assays. Immature fruit (0.5 to 1.5 cm) showed no *HMG2*:GUS expression. However, larger fruit (3-5 cm), mature green fruit (full size but without carotenoids), breaker fruit (undergoing carotenogenesis) and fully ripe fruit showed GUS activity. Histochemical analyses identified that the GUS activity was localized primarily to the developing seeds and fruit vasculature. The fruit showed dramatic wound-inducibility of the *HMG2*:GUS activity. *HMG2*:GUS activity was also localized to the developing seeds within the seed pods of tobacco.

Distinct regulatory circuits may mediate inducible versus developmental control of *HMG2* expression. During anther (organ producing male gametophyte) development in immature flowers, wounding induces high levels of GUS, but immature pollen show no activity. As pollen matures, the pollen expresses GUS but anthers no longer show wound-inducible activity.

6.2.3 DEFENSE-RELATED ACTIVITY OF THE *HMG2* PROMOTER

Wounding by excision or crushing triggered rapid increases in GUS activity within 1-12 hours. This response was seen in all tissues tested with the exception of the mature anthers of tobacco (discussed above). Tissues showing marked wound-induced activation of the *HMG2*:GUS construct include, but are not limited to, roots, hypocotyls, and leaves of tobacco and tomato seedlings; roots, stems, petioles, pedicles, all regions of expanding and mature leaves, developing fruit and pods, mature fruit and pods, and petals of mature tobacco and tomato plants.

Inoculation of seedlings or excised leaves with compatible bacterial pathogens triggered specific *HMG2*:GUS expression localized to the cells directly surrounding the bacterial lesion. This response was significant at 24 hours post-inoculation (the earliest time monitored) as shown in Figure 11, Panel A. The fungal pathogen, *Rhizoctonia solani*, also triggered dramatic increases in *HMG2*:GGS activity (Figure 11, Panel B) in inoculated seedlings of both tobacco and tomato. Because these widely different microbial pathogens both trigger very similar *HMG2* activation events, it is likely that other bacterial and fungal pathogens will elicit a similar response. At later times during *Rhizoctonia* infection, the fungus had spread down the vascular system of the plants and was associated with significant *HMG2*:GUS activity in these tissues. This suggests that the defense-activation properties of *HMG2* will function against vascular pathogens (e.g., wilt-inducing pathogens) as well.

6.2.4 NEMATODE ACTIVATION OF THE *HMG2* PROMOTER

Transgenic plants containing the 2.3 kb *HMG2* promoter fused to GUS were analyzed for GUS activity following nematode inoculation. Tomato seedlings, germinated axenically on agar medium, were inoculated by addition of second-stage juveniles of either *Meloidogyne incognita* or *M. hapla*, causal agents of root knot disease. The transgenic cultivars were susceptible to both species. Prior to inoculation and at various times following addition of juveniles, roots were removed, histochemically assayed for GUS activity, and stained for nematodes using acid fucsin. Uninoculated roots and roots at 24 and 48 hours after inoculation showed no GUS activity on root tips (the site of nematode penetration), but GUS activity was evident at the zone of lateral root initiation and at the wound site where the stem was removed. At 24 and 48 hours post-inoculation, roots containing juveniles were observed which showed no indication of GUS activity (Figure 12, Panel A). By 72 hrs post-inoculation some roots showed low levels

of GUS activity in cells adjacent and proximal (toward the stem) to the juvenile (Figure 12, Panel B). By five days post-inoculation, high levels of GUS activity was evident surrounding nematodes that has initiated feeding (characterized by a change in nematode morphology). By days 5-7, infected root tips showed visible swelling. This galled tissue expressed extremely high levels of GUS (Figure 12, Panels C, D and E). The responses to *M. incognita* and *M. hapla* did not differ significantly. Observation of multiple roots and plant samples suggested that the onset of *HMG2* activation was closely associated with the establishment of feeding behavior. This suggests that utilization of this promoter to drive expression of transgene proteins which are toxic or inhibitory to nematode development would significantly impact disease development. Both the temporal and spacial expression pattern are optimal for rapid and highly localized delivery of nematocides or nematostatic agents.

6.2.5 ACTIVATION OF THE *HMG2* PROMOTER BY NATURAL PREDATORS

Transgenic tobacco plants grown in the field were tested for *HMG2*:GUS expression in response to natural predation. Leaf sections surrounding sites of localized necrosis due to insect or microbial damage were excised and tested for localized expression of *HMG2*:GUS. Leaf tissue that had not been damaged showed no GUS activity. However, the region immediately surrounding the lesion showed intense GUS staining (Figure 11, Panel C). Transgenic tobacco plants were planted in Blackstone, VA in fields known to provide high levels of disease pressure due to the tobacco cyst nematode (*Globodera tobacum* ssp. *solanaceara*) in order to assess *HMG2* responses to natural nematode infection.

6.2.6 DISSECTION OF *HMG2* PROMOTER

In order to localize elements mediating defense- and tissue-specific expression, efforts were made to generate a

series of 5' promoter deletions. In the central region of the *HMG2* promoter, delineated in Figure 5, are several stretches of ATs which seemed to prevent exonuclease digestion through this area. After repeated unsuccessful attempts at exonuclease strategies, an alternative strategy based on PCR was used to generate deletions of the *HMG2* promoter (see Figure 7). These truncated promoters were fused to GUS reporter genes in the plant expression plasmid pBI101 (Clontech) and introduced into tobacco. The promoter region from -981 to +110 confers both wound and microbial pathogen responses as well as trichome and pollen expression (Table 4). However, analyses of multiple independently transformed tobacco plants suggest that the levels of transgene expression driven by this promoter are less than that of promoters that are larger (e.g., to -2.3 kb) or smaller (e.g., -347 bp). This suggests that this region may contain a "silencer" and that the region upstream of 891 contains an additional enhancer-type element. All deletions, including the construct containing only 58 bp of *HMG2* upstream of the transcriptional start site continue to drive transgene expression in the pollen suggesting that developmental regulation is distinct from that associated with defense.

TABLE 4: DISSECTION OF TOMATO *HMG2* PROMOTER ELEMENT

HMG2:GUS CONSTRUCT	PROMOTER[a] REGION (bp)	INDUCED AND TISSUE EXPRESSION[b] Wound	Pathogenc	Trichomes	Pollen	
SLJ 330.1	-2.3k to +118	++	+++	++	++	–
DW 203	-891 to +125	+	+	+	++	
DW 202	-347 to +125	++	++	++	++	
DW 201	-58 to +125	--	--	--		+

a Numbering based on the transcriptional start site designated as +1. The ATG start codon begins at base 111. GUS fusions were translationally in frame and added several bases of *HMG2* coding region to the N-terminus of the GUS gene.

b GUS phenotype assayed in multiple independently-transformed plants for each construct.

c Based on leaf assays following inoculation with the soft-rotting bacterium *Erwinia carotovora* ssp. *carotovora*.

6.2.7 *HMG2* CIS-REGULATORY ELEMENTS

Specific *cis*-regulatory elements within the *HMG2* promoter are identified based on functional analyses and DNA-protein interactions. For example, the region between bases -58 and -347 contains one or more elements which direct strong wound and pathogen induction. The -58 to -347 fragment is generated by PCR using primer 21 and a primer complementary to primer 19 (see Figure 4). Additional 3' and 5' oligonucleotide primers synthesized based on sequences within this region will generate subfragments for analyses of regulatory activity. Functional analyses entail fusion of these fragments to appropriate "minimal" plant promoters fused to a reporter gene such as the minimal 35S promoter (discussed in Sections 5.2 and 5.3, above) and introduction and expression of the resulting constructs in plants or plant cells. Regions containing fully-functional *HMG2* cis-regulatory elements will confer *HMG2*-specific regulation on this minimal promoter as determined by wound-, pathogen-, pest-, or elicitor-inducibility or generation of *HMG2* sequence-dependent developmental patterns of GUS expression. Specific cis-regulatory elements are further delineated by gel shift or footprint/DNase sensitivity assays. The -58 to -347 fragment or other *HMG2* promoter fragments, generated by PCR amplification, are incubated with nuclear extracts from control and defense-elicited cells. For example, nuclei are isolated (Elliot et al., 1985, Plant Cell 1:681-690) from untreated tomato suspension cultured cells and cells 6-8 hours after treatment with fungal elicitor or arachidonic acid (Park, 1990, *Lycopersican esculentum* Mill., Ph.D. Dissertation, Virginia Polytechnic Institute and State University; Yang et al., 1991, Plant Cell 3:397-405). PCR fragments which interact with nuclear components are identified based on reduced mobility in polyacrylamide gel electrophoresis or by digestion with DNase I and analysis of DNase-insensitive areas on sequencing gels. Defense-responsive elements may or may not demonstrate differential patterns upon interaction with control versus elicitor-treated

extracts. Promoter regions identified as having DNA-protein interactions are further tested by creating chimeric construct with, for example, "minimal" 35S promoters to determine effects of the cis-regulatory element on heterologous gene expression in transgenic plants or plant cells. This strategy may separate specific *HMG2* promoter functions (e.g., wound from pathogen induction, or defense from developmental regulation) and delineate additional or novel uses of *HMG2* promoter elements.

6.2.8. USE OF *HMG2* PROMOTER FOR DEVELOPING NEMATODE RESISTANT PLANTS

Another important use of the present invention is the development of namatode-resistant plants. Such resistant plants is developed by using an *HMG2* promoter element to control the expression of a heterologous gene which is or whose product is toxic or inhibitory to nematodes. Key advantages of using the *HMG2* expression systems for this purpose are that 1) the heterologous gene product will be limited primarily to tissues undergoing disease-related stress, and 2) the heterologous gene will be strongly activated directly in the tissue of ingress, thus delivering a significant "dose" to the nematode.

The coding region of potato proteinase inhibitor I or II (Johnson et al., 1989, Proc. Natl. Acad. Sci. USA 86:9871-9875) is excised by appropriate restriction enzymes and inserted into a plant expression vector downstream of the *HMG2* promoter or a chimeric promoter containing active *cis*-regulatory element(s) of the *HMG2* promoter. Appropriate enzyme sites or linker fragments are used to assure correct positioning of the coding region with the transcriptional and translational initiation sites. The plasmid pDW202 (Figure 7) is digested with SmaI and SstI and gel purified to obtain the vector/*HMG2* promoter fragment separate from the fragment containing the excised GUS coding region. The appropriately digested and processed potato proteinase inhibitor I gene is then ligated into the SmaI/SstI site. The resulting plasmid

is transformed into *E coli* strain DH5α and sequenced to assure correct insertion and orientation prior to introduction into *Agrobacterium* strain LBA4404 by triparental mating. The engineered LBA4404 is then used for tobacco leaf disk co-cultivation and the transformed tobacco is subsequently regenerated to mature plants.

Enzymatic or immuno-detection of proteinase inhibitor I in transgenic plants is done by described methods (Johnson et al., 1989, Proc. Natl. Acad. Sci. USA 86:9871-9875). The effect of the introduced *HMG2*:Proteinase inhibitor constructs on plant:nematode interactions is tested in seedlings inoculated, for example, with root knot (*Meloidogyne incognita, M. halpa*) or cyst (*Globodera tobacum*) nematodes or by plant seedlings in fields of known disease pressure. Plants are monitored for overall health, number of feeding female nematodes, number and severity of galls, and nematode egg production.

In another strategy, *HMG2* promoter-driven expression of an anti-sense RNA may be used to block production of plant compounds required by parasitic nematodes. For instance, root knot and cyst nematodes are dependent upon plant sterols for growth and reproduction. Thus, sequences from the tomato *HMG1* (sterol-specific) isogene (e.g., regions with the N-terminus specific for *HMG1* and not other HMGR isogenes) or the tomato squalene synthetase gene are introduced into the SmaI/SstI digested vector described above such that the complementary RNA strand is produced. Expression of these gene constructs in transgenic tomato decreases or blocks sterol production in plant cells where *HMG2* promoter is active and thus reduces or prevents nematode growth and development.

7.0. EXAMPLE: POST-HARVEST PRODUCTION USING AN EXPRESSION CONSTRUCT COMPRISING THE TOMATO *HMG2* OPERABLY LINKED TO GENE OF INTEREST

Post-harvest production of desired gene products in harvested plant tissues and cultures depends on the ability of the harvested material to remain competant for induced gene

expression. The experiments described below demonstrate such an ability in harvested leaf tissue. Moreover, in the harvested leaf the *HMG2* promoter activity superior to that of the often used constitutive CaMV 35 promoter.

The plants used in these experiments are from the *HMG2*:GUS or CaMV 35S:GUS transformed tobacco plants described in section 6.2.1. above.

7.1. *HMG2* PROMOTER IS SUPERIOR TO CaMV 35S PROMOTER IN POST-HARVEST PRODUCTION

The yield of a specific transgene. β-glucuronidase (GUS), was compared for transgenic plants containing the *HMG2*:GUS construct versus those containing a 35S:GUS construct. The 35S promoter from the cauliflower mosaic virus is widely used in transgenic plant research because it directs high levels of transgene expression in most tissues (Benfey et al., 1989, EMBO J. 8:2195-2202). Leaf material was harvested from greenhouse-grown plants and wounded by scoring with a razor blade. Tissue was extracted for GUS activity immediately after harvest/wounding and after 24 and 48 hours of room temperature storage in zip-lock bags to prevent desiccation. As shown in Figure 13, the leaves containing the 35S:GUS construct showed greater that 50% loss of GUS activity within 24 hours of harvest. For industrial utilization of transgenic tobacco for bioproduction of a high-value protein, this loss would be highly significant. In contrast, the *HMG2*:GUS construct was less active immediately after harvest, but by 48 hours, directed wound-induced transgene product accumulations to levels comparable to pre-storage 35S:GUS levels.

A transgenic plant carrying a heterologous gene encoding a valuable product, for example a human therapeutic protein of pharmaceutical value, fused to the *HMG2* promoter would be expressed at relatively low levels throughout the growing cycle of a transgenic plant (e.g., tobacco). Thus, transgene expression is unlikely to affect biomass yield. Upon optimal biomass production, the plants are harvested

(e.g., by mowing) and the leaf material is transferred to the laboratory or processing facility. HMG2-driven heterologous gene expression triggered by mechanical wounding or wounding plus chemical or elicitor treatment and the transgene product is then recovered for further processing accumulation. The time range of product accumulation is generally (but not limited to) 24 to 72 hours depending on the time and conditions between harvest and induction and the stability properties of the specific heterologous gene product.

Heterologous gene products whose production or high-level accumulation is deleterious to the growth or vigor of the plant can similarly be produced by utilizing the post-harvest induction characteristics of the *HMG2* promoter. For highly toxic products it is advantageous to further dissect the promoter to minimize developmental expression but retain the wound and elicitor inducible responses. Promoter deletion analyses (Table 4) indicate that multiple *cis*-regulatory elements are present in the promoter and that the developmental functions (e.g., pollen) are separable from the defense-specific functions (e.g., wound, pathogen responses). These analyses indicate that the wound and pathogen response elements are distal to those elements mediating pollen expression.

7.2. HARVESTED PLANT TISSUE MAINTAIN INDUCIBLE GENE EXPRESSION

The usefulness of post-harvest production of desired gene products depends in part on the ability of the harvested plant tissue or culture to maintain inducible gene expression for some time after harvest. Such an ability means that the method of invention would have enhanced industrial utility in as much as it may not always be practical to induce and process the plant tissue or culture immediately after harvesting the material. For example, the harvested material may require transportation to a distant site for induction or processing.

Leaves from grown tobacco plants engineered with the *HMG2*:GUS construct were harvested and either induced immediately or after 2 weeks of storage at 4°C in sealed containers. The results shown in Figure 14 indicate that the stored leaves not only remained competant for the induced expression of *HMG2*:GUS construct but actual expressed nearly 40-50% more activity than freshly harvested leaves. Figure 14 also shows that the induced material requires some incubation time, up to 48 hr., at room temperature to allow for expression of the induced gene product.

Figure 15 shows that the harvested leaves can be stored either at 4°C in sealed containers or at room temperature in air permeable containers for up to 6 weeks and still have ability equal to that of the freshly harvested material for the expression of inducible genes.

Although the invention is described in detail with reference to specific embodiments thereof, it will be understood that variations which are functionally equivalent are within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein, the disclosure of which are incorporated by reference in their entireties.

CLAIMS

1. A recombinant DNA molecule containing a *HMG2* promoter element, or a functional portion thereof.

2. The recombinant DNA molecule according to claim 1, wherein the *HMG2* promoter element is the tomato *HMG2* promoter or a sequence that selectively hybridizes to the tomato *HMG2* promoter.

3. The recombinant DNA molecule according to claim 1, wherein the *HMG2* promcter element comprises the approximately 2.3 kb EcoRI-BglII restriction fragment of pDW101, a functional portion of said fragment or a sequence that selectively hybridizes to said fragment.

4. The recombinant DNA molecule according to claim 1, wherein the *HMG2* promoter element comprises the approximately 0.17 kb HindIII-BamHI restriction fragment of pDW201, a functional portion of said fragment, or a sequence that selectively hybridizes to said fragment.

5. The recombinant DNA molecule according to claim 1, wherein the *HMG2* promoter element comprises the approximately 0.46 kb HindIII-BamHI restriction fragment of pDW202, a functional portion of said fragment, a sequence that selectively hybridizes to said fragment.

6. The recombinant DNA molecule according to claim 1, wherein the *HMG2* promoter element comprises the approximately 1.0 kb HindIII-BamHI restriction fragment of pDW203, a functional portion of said fragment, a sequence that selectively hybridizes to said fragment.

7. A gene fusion comprising a *HMG2* promoter element, or a functional portion thereof, operably linked to a second nucleotide sequence encoding a heterologous gene, wherein the transcription of the heterologous gene is under the control of said *HMG2* promoter element.

8. The gene fusion according to claim 7, wherein the *HMG2* promoter element is the tomato *HMG2* promoter or a sequence that selectively hybridizes to the tomato *HMG2* promoter.

9. The gene fusion according to claim 7, wherein the *HMG2* promoter element comprises the approximately 2.3 kb EcoRI-BglII restriction fragment of pDW101, a functional portion of said fragment or a sequence that selectively hybridizes to said fragment.

10. The gene fusion according to claim 7, wherein the *HMG2* promoter element comprises the approximately 0.17 kb HindIII-BamHI restriction fragment of pDW201, a functional portion of said fragment, or a sequence that selectively hybridizes to said fragment.

11. The gene fusion according to claim 7, wherein the *HMG2* promoter element comprises the approximately 0.46 kb HindIII-BamHI restriction fragment of pDW202, a functional portion of said fragment, a sequence that selectively hybridizes to said fragment.

12. The gene fusion according to claim 7, wherein the *HMG2* promoter element comprises the approximately 1.0 kb HindIII-BamHI restriction fragment of pDW203, a functional portion of said fragment, a sequence that selectively hybridizes to said fragment.

13. A chimeric promoter comprising a plant promoter or truncated promoter ligated to a sequence containing a cis-regulatory element of a *HMG2* promoter, wherein

i) the truncated promoter is not derived from said *HMG2* promoter,

ii) the sequence is ligated 5' upstream of truncated promoter or within the regulatory region of the plant promoter, and

iii) the transcriptional activity of the chimeric promoter is regulated by said sequence.

14. The chimeric promoter of claim 13, wherein the *HMG2* promoter is the tomato *HMG2* promoter.

15. The chimeric promoter of claim 13, wherein the cis-regulatory element containing sequence comprises the tomato *HMG2* promoter sequence depicted in Figure 4 from nucleotide residue number -35 to -1,036, a portion of said sequence or a sequence that selectively hybridizes to said sequence depicted in Figure 4.

16. The chimeric promoter of claim 13, wherein the cis-regulatory element containing sequence comprises the 480 bp tomato *HMG2* promoter sequence depicted in Figure 5, a portion of said sequence or a sequence that selectively hybridizes to said sequence depicted in Figure 5.

17. The chimeric promoter of claim 13, wherein the cis-regulatory element containing sequence comprises the 515 bp sequence depicted in Figure 6, a portion of said sequence or a sequence that selectively hybridizes to said sequence depicted in Figure 6.

18. A gene fusion comprising the chimeric promoter of claim 13 operably linked to a second nucleotide sequence encoding a heterologous gene, wherein the transcription of the gene is under the control of said chimeric promoter.

19. A gene fusion comprising the chimeric promoter of claim 14 operably linked to a second nucleotide sequence encoding a heterologous gene, wherein the transcription of the gene is under the control of said chimeric promoter.

20. A gene fusion comprising the chimeric promoter of claim 15 operably linked to a second nucleotide sequence

encoding a heterologous gene, wherein the transcription of the gene is under the control of said chimeric promoter.

21. A gene fusion comprising the chimeric promoter of claim 16 operably linked to a second nucleotide sequence encoding a heterologous gene, wherein the transcription of the gene is under the control of said chimeric promoter.

22. A gene fusion comprising the chimeric promoter of claim 17 operably linked to a second nucleotide sequence encoding a heterologous gene, wherein the transcription of the gene is under the control of said chimeric promoter.

23. A recombinant DNA vector containing the gene fusion of any of claims 7 to 12 and 18 to 22.

24. A plant cell culture, wherein the plant cells of said culture contain the gene fusion of any of claims 7 to 12 and 18 to 22.

25. A plant containing the gene fusion of any of claims 7 to 12 and 18 to 22.

26. A method for producing a product encoded by a heterologous gene in plant cell culture, comprising cultivating the plant cell culture of claim 24; inducing the expression of the gene fusion in the transformed plant cells by wounding or elicitor treatment; and recovering the expressed heterologous gene product from the transformed plant cells.

27. A method for producing a product encoded by a heterologous gene in plant, comprising growing the plant of claim 25; inducing the expression of the gene fusion in the plant by wounding, elicitor treatment, pathogen infection, or pest infestation; harvesting the plant; and processing the

plant material for use or extracting the expressed heterologous gene product from the plant.

28. The method of 27, wherein the expression of the gene fusion is induced immediately prior to, during or immediately after harvesting the plant.

29. The plant of claim 25, wherein the heterologous gene of said gene fusion encodes a microbial disease resistance gene and the plant is resistant to a microbial disease due to the expression of said resistance gene under the control of the *HMG2* promoter element or the chimeric promoter comprising a *HMG2* cis-regulatory element.

30. The plant of claim 25, wherein the heterologous gene of said gene fusion encodes a pest resistance gene and the plant is resistant to a plant pest due to the expression of said resistance gene under the control of the *HMG2* promoter element or the chimeric promoter comprising a *HMG2* cis-regulatory element.

31. A method for producing a product encoded by a heterologous gene in a plant cell, comprising

a) constructing an expression vector comprising an inducible promoter element, or a functional portion thereof, operably linked to a second nucleotide sequence encoding a heterologous gene, wherein said inducible promoter element or a functional portion of said element controls the transcription of said heterologous gene sequence;

b) engineering a plant cell with said expression vector;

c) culturing said engineered plant cell;

d) harvesting said engineered plant cell; and

e) inducing the expression of said heterologous gene; and

f) recovering the expressed heterologous gene product from said engineered plant cell.

32. A method for producing a product encoded by a heterologous gene in harvested plant tissue, comprising

a) constructing an expression vector comprising an inducible promoter element, or a functional portion thereof, operably linked to a second nucleotide sequence encoding a heterologous gene, wherein said inducible promoter element or a functional portion of said element controls the transcription of said heterologous gene sequence;

b) engineering a plant with said expression vector;

c) cultivating said engineered plant;

d) harvesting tissue from said engineered plant; and

e) inducing the expression of said heterologous gene; and

f) recovering the expressed heterologous gene product from said engineered plant.

33. The method of claim 31 or 32, wherein said inducible promoter element is a *HMG2* promoter.

34. The method of claim 33, wherein said inducible promoter is a tomato *HMG2* promoter or a tomato *HMG2* promoter homolog.

35. The method of claim 33, wherein inducing the expression of said heterologous gene is by wounding or elicitor treatment.

36. The method of claim 34, wherein inducing the expression of said heterologous gene is by wounding or elicitor treatment.

37. The method of claim 32, wherein said harvested tissue is leaf, stem, root, flower, seed or fruit.

38. The method of claim 37, wherein said harvested tissue is leaf.

AMENDED CLAIMS

[received by the International Bureau on 16 January 1995 (16.01.95); original claim 4 cancelled; original claims 1-3 amended; claims 5,6,8-13,15-17, 23-25,29 and 30 amended and renumbered as claims 4,5,7-12,14-16,22-24,28 and 29; other claims unchanged but renumbered accordingly (7 pages)]

1. A substantially pure *HMG2* promoter element or a functional portion thereof, wherein said *HMG2* promoter element is a plant promoter element, and said *HMG2* promoter element or said functional portion confers wound-induced, pathogen infection-induced, pest infestation-induced, chemical-induced or elicitor-induced expression to an operably linked coding sequence.

2. The substantially pure *HMG2* promoter element according to claim 1, wherein the *HMG2* promoter element is a tomato *HMG2* promoter or a promoter sequence that selectively hybridizes under high stringency conditions to the tomato *HMG2* promoter.

3. The substantially pure *HMG2* promoter element according to claim 2, wherein the *HMG2* promoter element comprises the 2.3 kb EcoRI-BglII restriction fragment of pDW101, a functional portion of said fragment or a promoter sequence that selectively hybridizes under high stringency conditions to said fragment.

4. The substantially pure *HMG2* promoter element according to claim 2, wherein the *HMG2* promoter element comprises the 0.46 kb HindIII-BamHI restriction fragment of pDW202, a functional portion of said fragment, or a promoter sequence that selectively hybridizes under high stringency conditions to said fragment.

5. The substantially pure *HMG2* promoter element according to claim 2, wherein the *HMG2* promoter element comprises the 1.0 kb HindIII-BamHI restriction fragment of pDW203, a functional portion of said fragment, or a promoter sequence that selectively hybridizes under high stringency conditions to said fragment.

6. A gene fusion comprising a *HMG2* promoter element, or a functional portion thereof, operably linked to

a second nucleotide sequence encoding a heterologous gene, wherein the transcription of the heterologous gene is under the control of said *HMG2* promoter element.

7. The gene fusion according to claim 6, wherein the *HMG2* promoter element is the tomato *HMG2* promoter or a promoter sequence that selectively hybridizes under high stringency conditions to the tomato *HMG2* promoter.

8. The gene fusion according to claim 7, wherein the *HMG2* promoter element comprises the 2.3 kb EcoRI-BglII restriction fragment of pDW101, a functional portion of said fragment, or a promoter sequence that selectively hybridizes under high stringency conditions to said fragment.

9. The gene fusion according to claim 7, wherein the *HMG2* promoter element comprises the 0.17 kb HindIII-BamHI restriction fragment of pDW201, a functional portion of said fragment, or a promoter sequence that selectively hybridizes under high stringency conditions to said fragment.

10. The gene fusion according to claim 7, wherein the *HMG2* promoter element comprises the 0.46 kb HindIII-BamHI restriction fragment of pDW202, a functional portion of said fragment, a promoter sequence that selectively hybridizes under high stringency conditions to said fragment.

11. The gene fusion according to claim 7, wherein the *HMG2* promoter element comprises the 1.0 kb HindIII-BamHI restriction fragment of pDW203, a functional portion of said fragment, or a promoter sequence that selectively hybridizes under high stringency conditions to said fragment.

12. A chimeric promoter comprising a plant promoter or truncated promoter ligated to a first sequence having a cis-regulatory element of a plant *HMG2* promoter, wherein

i) the truncated promoter comprises a second sequence whose 3'-end is at the nucleotide corresponding to the transcription initiation site of said truncated promoter and whose 5'-end is at a nucleotide 46 to 150 bases 5' upstream of said 3'-end, and which truncated promoter is not derived from said *HMG2* promoter,

ii) the first sequence is ligated 5' upstream of the truncated promoter or within the regulatory region of the plant promoter, and

iii) the transcriptional activity of the chimeric promoter is regulated by said first sequence.

13. The chimeric promoter of claim 12, wherein the *HMG2* promoter is the tomato *HMG2* promoter.

14. The chimeric promoter of claim 12, wherein the cis-regulatory element comprises the tomato *HMG2* promoter sequence depicted in Figure 4 from nucleotide residue number -35 to -1,036, a portion of said sequence, or a promoter sequence that selectively hybridizes under high stringency conditions to said sequence depicted in Figure 4.

15. The chimeric promoter of claim 12, wherein the cis-regulatory element comprises the 480 bp tomato *HMG2* promoter sequence depicted in Figure 5, a portion of said sequence, or a promoter sequence that selectively hybridizes under high stringency conditions to said sequence depicted in Figure 5.

16. The chimeric promoter of claim 12, wherein the cis-regulatory element comprises the 515 bp sequence depicted in Figure 6, a portion of said sequence, or a promoter sequence that selectively hybridizes under high stringency conditions to said sequence depicted in Figure 6.

17. A gene fusion comprising the chimeric promoter of claim 12 operably linked to a second nucleotide sequence encoding a heterologous gene, wherein the transcription of the gene is under the control of said chimeric promoter.

18. A gene fusion comprising the chimeric promoter of claim 13 operably linked to a second nucleotide sequence encoding a heterologous gene, wherein the transcription of the gene is under the control of said chimeric promoter.

19. A gene fusion comprising the chimeric promoter of claim 14 operably linked to a second nucleotide sequence encoding a heterologous gene, wherein the transcription of the gene is under the control of said chimeric promoter.

20. A gene fusion comprising the chimeric promoter of claim 15 operably linked to a second nucleotide sequence encoding a heterologous gene, wherein the transcription of the gene is under the control of said chimeric promoter.

21. A gene fusion comprising the chimeric promoter of claim 16 operably linked to a second nucleotide sequence encoding a heterologous gene, wherein the transcription of the gene is under the control of said chimeric promoter.

22. A recombinant DNA vector having the gene fusion of any of claims 6 to 11 and 17 to 21.

23. A plant cell culture, wherein the plant cells of said culture have the gene fusion of any of claims 6 to 11 and 17 to 21.

24. A plant having the gene fusion of any of claims 6 to 11 and 17 to 21.

25. A method for producing a product encoded by a heterologous gene in plant cell culture, comprising cultivating the plant cell culture of claim 23; inducing the

expression of the gene fusion in the transformed plant cells by wounding or elicitor treatment; and recovering the expressed heterologous gene product from the transformed plant cells.

26. A method for producing a product encoded by a heterologous gene in plant, comprising growing the plant of claim 24; inducing the expression of the gene fusion in the plant by wounding, elicitor treatment, pathogen infection, or pest infestation; harvesting the plant; and processing the plant material for use or extracting the expressed heterologous gene product from the plant.

27. The method of 26, wherein the expression of the gene fusion is induced immediately prior to, during or immediately after harvesting the plant.

28. The plant of claim 24, wherein the heterologous gene of said gene fusion encodes a microbial disease resistance gene.

29. The plant of claim 24, wherein the heterologous gene of said gene fusion encodes a pest resistance gene.

30. A method for producing a product encoded by a heterologous gene in a plant cell, comprising

a) constructing an expression vector comprising an inducible promotor element, or a functional portion thereof, operably linked to a second nucleotide sequence encoding a heterologous gene, wherein said inducible promoter element or a functional portion of said element controls the transcription of said heterologous gene sequence;

b) engineering a plant cell with said expression vector;

c) culturing said engineered plant cell;

d) harvesting said engineered plant cell;
e) inducing the expression of said heterologous gene; and
f) recovering the expressed heterologous gene product from said engineered plant cell.

31. A method for producing a product encoded by a heterologous gene in harvested plant tissue, comprising
a) constructing an expression vector comprising an inducible promotor element, or a functional portion thereof, operably linked to a second nucleotide sequence encoding a heterologous gene, wherein said inducible promoter element or a functional portion of said element controls the transcription of said heterologous gene sequence;
b) engineering a plant with said expression vector;
c) cultivating said engineered plant;
d) harvesting tissue from said engineered plant;
e) inducing the expression of said heterologous gene; and
f) recovering the expressed heterologous gene product from said engineered plant cell.

32. The method of claim 30 or 31, wherein said inducible promotor element is a *HMG2* promoter.

33. The method of claim 32, wherein said inducible promotor element is a tomato *HMG2* promoter or a tomato *HMG2* promoter homolog.

34. The method of claim 32, wherein inducing the expression of said heterologous gene is by wounding or elicitor treatment.

35. The method of claim 33, wherein inducing the expression of said heterologous gene is by wounding or elicitor treatment.

36. The method of claim 31, wherein said harvested tissue is leaf, stem, root, flower, seed, or fruit.

37. The method of claim 36, wherein said harvested tissue is leaf.

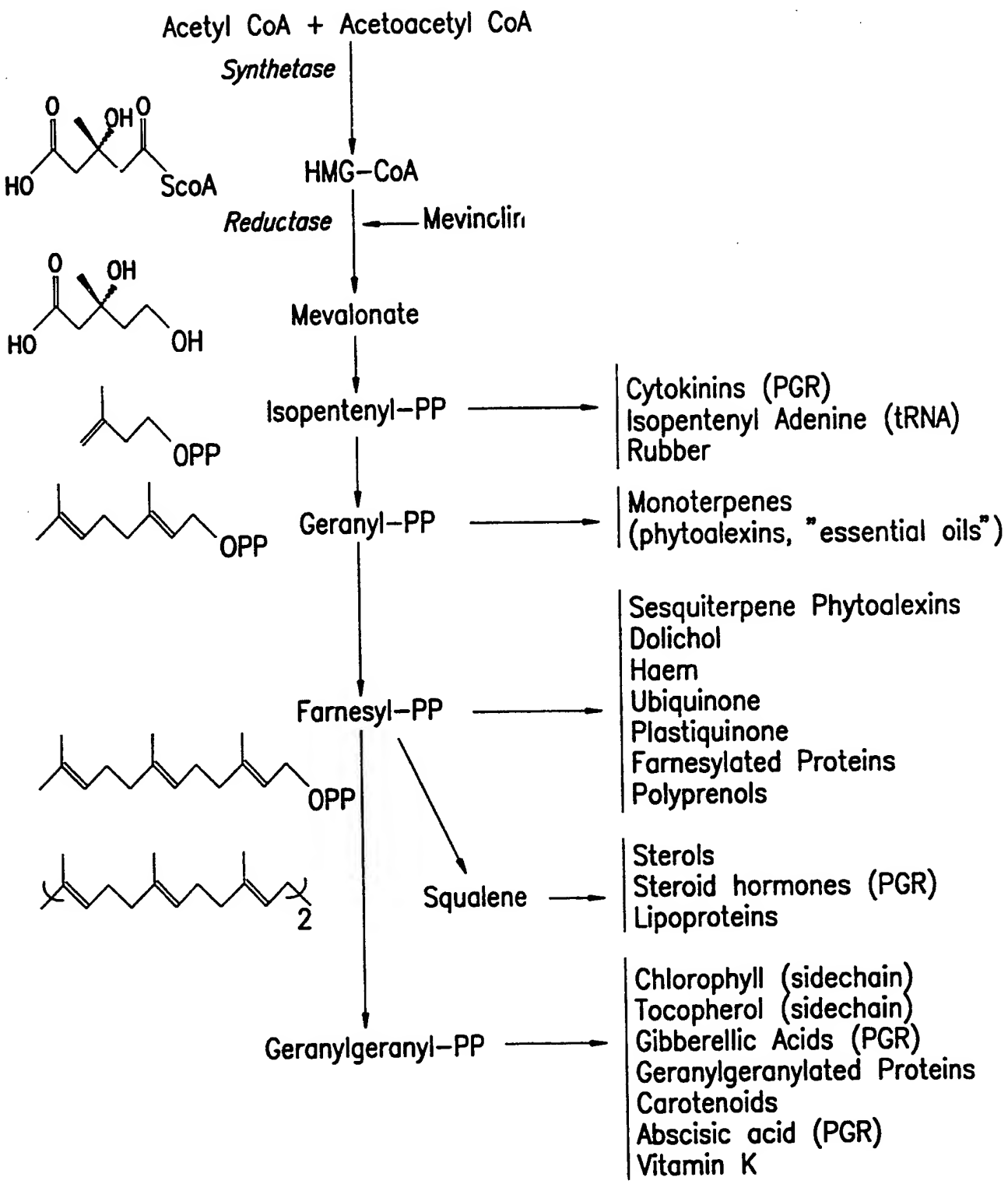

FIG.1

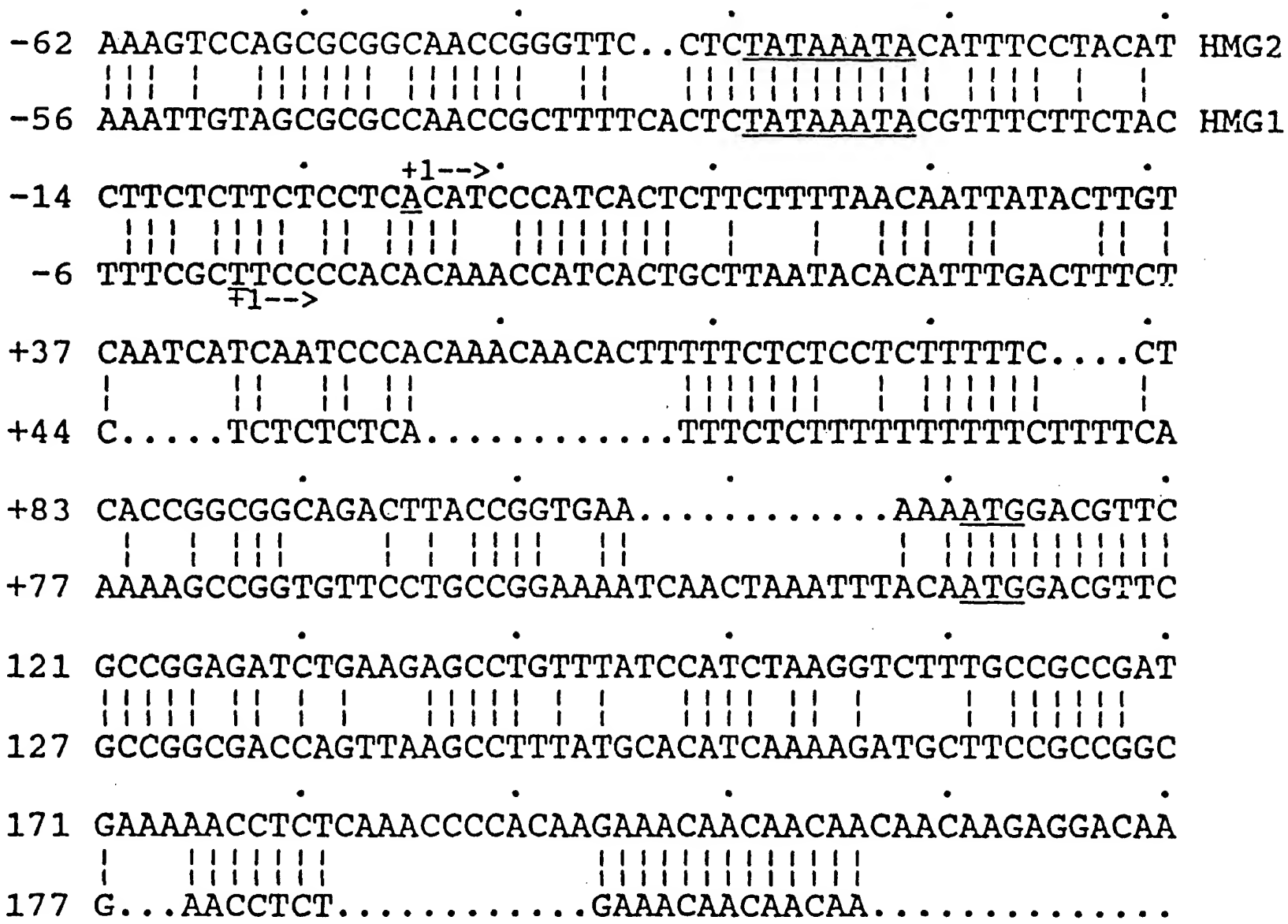

FIG. 2A

FIG. 2B

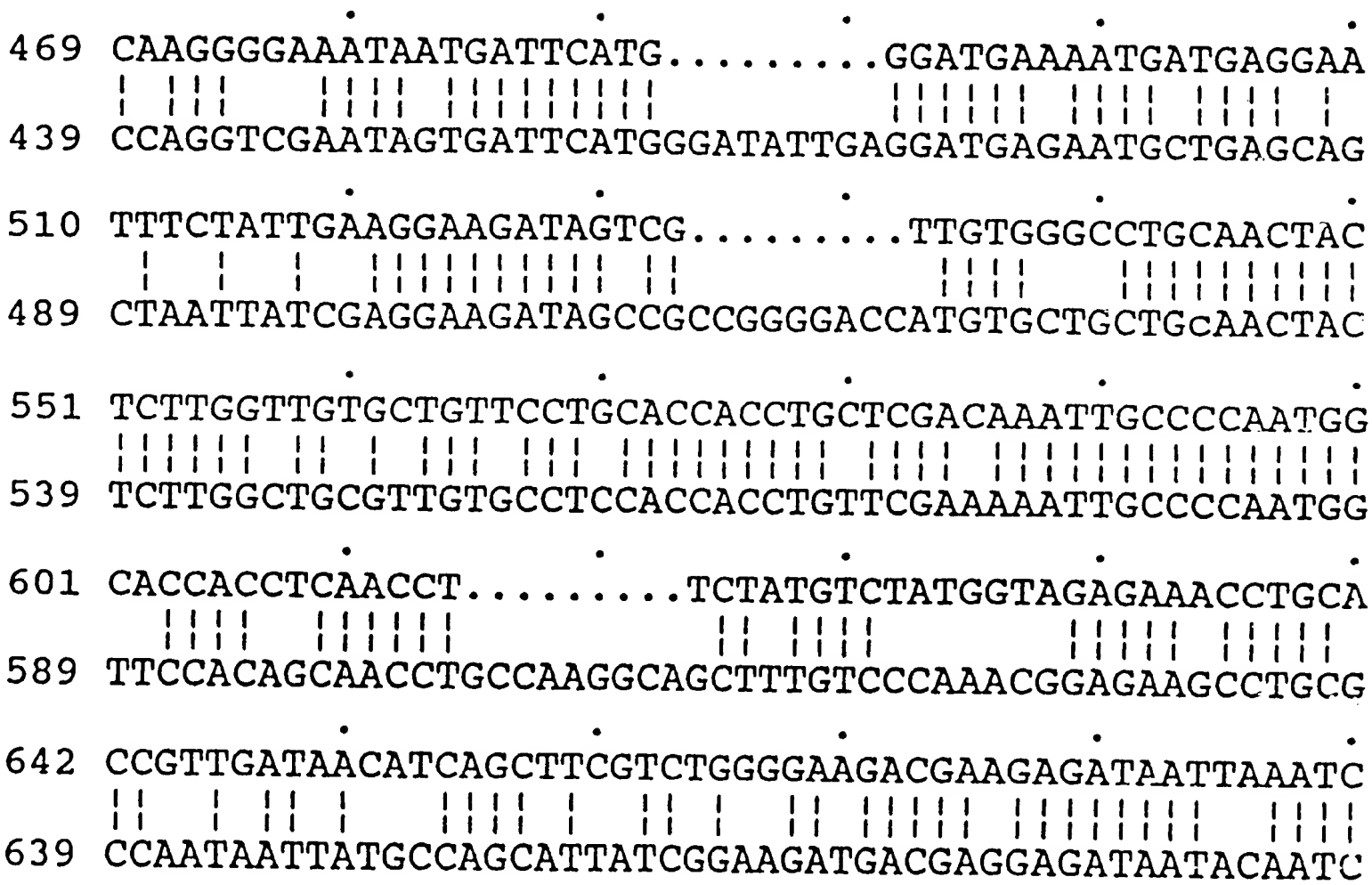

FIG.2C

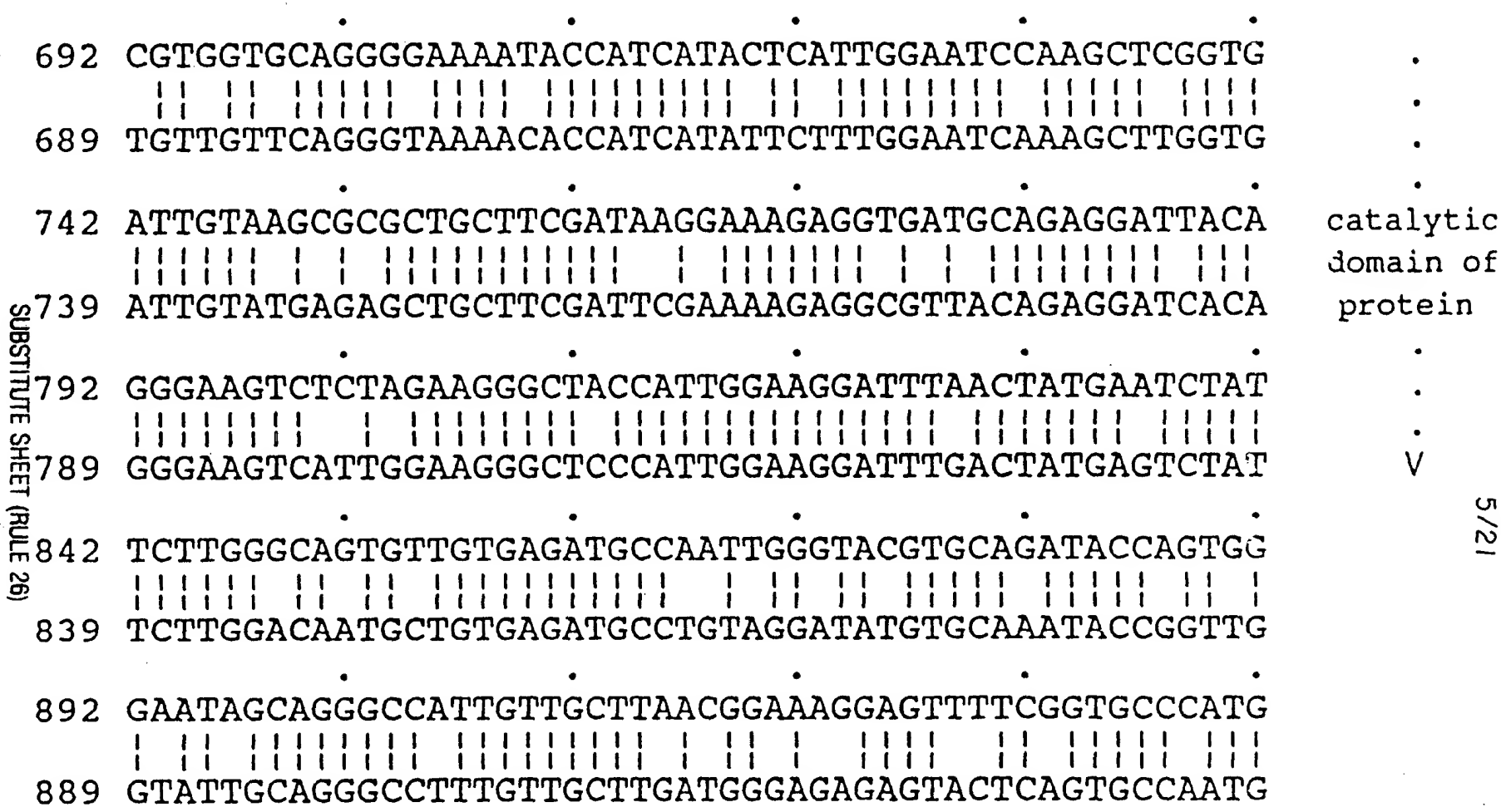

FIG. 2D

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942  GCAACCACACAGAAGGATGTTTAGTGGCTAGCACCAACAGAGGTTGCAAGGC
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
939  GCAACTACAGAAGGGTGTTTAGTTGCTAGCACCAACAGGGGGTTGCAAGGC

992  TATCTATGCTTCTGGTGGTGCTACATGCATTTTTGCTTCGTGATGGTATGA
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
989  TATCTTTTGTCTCTGGTGGCGCCCAACAGCATTTTTGCTCAGAGATGGCATGA

1042 CCAGAGGCACCCATGTGTCAGGTTCGGCACAGCCAAAAAGGGCAGCAGAGTTG
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
1039 CAAGAGCTCCCGGTTGTCCGGTTCACCACCGCCAAAAAGAGCCGCAGAGTTG

1092 AAGTTCTTTGTTGAAGATCCCATAAAATTTGAGTCACTTGCTAACGTTTTT
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
1089 AAATTCTTCGTTGAGGATCCCCCTTAACTTTGAGATTCTTTCCCTTATGTT

1142 CAACCAGTAAGT end of Intron I
     ||||||||||||
1139 CAACAAGTGAGT end of Intron I
```

FIG. 2E

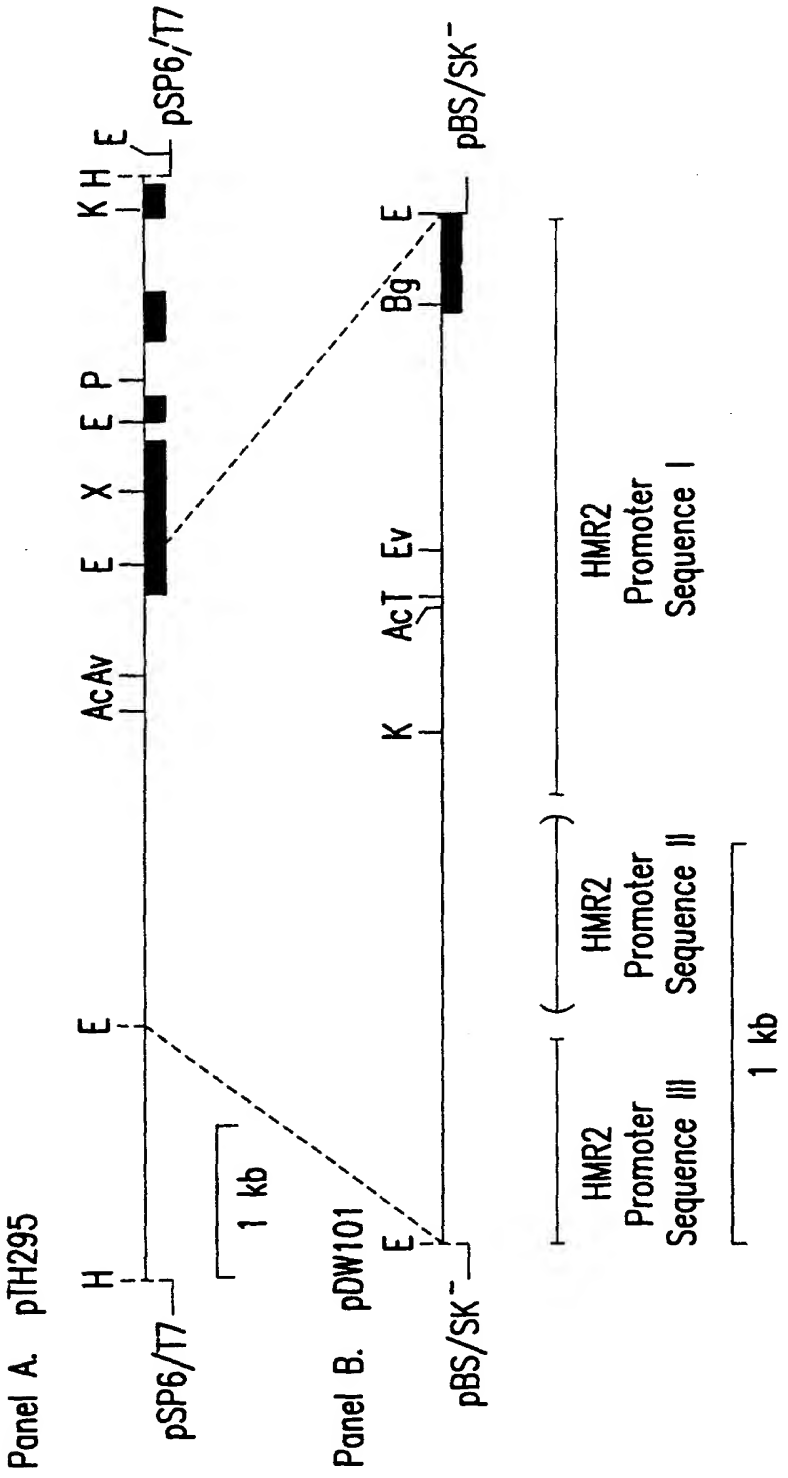

FIG.3

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-1036  AAATTgcTGA GATACCTCCT TTGTCTTTTT TCCACTGTGG TCTCAGTTGA
 -986  CTATAAGAAA TGTTGCTAAC TATTTTTAAT GTCCAAACCT CAAATATCAA
 -936  GCCATGCAAA ATTAACCATT TTCTTAACGT GGATATATAC CTATTCCACC
 -886  ACATGGTACC CTATTCCAAC TATATTAAAA AAAATAAAAT ACTCTAGTTA
            primer 22
 -836  ATATTGATCA AAATTTATAT AATTTACATC TCAATAATAT AAAAAGTTAT
 -786  TGCATGCGTA CTTATGAAAT TTGTAGGTTT TAAAATTGTG TAGGGCTGGG
 -736  CATAGAAAAT TGAATTATCA GATCGGATGG ACAGTTTTTT GGTATTTGGT
 -686  ATTCGGTAAT TAAGTATTTA ATATGATATT TGGAAGTACA ATTTTAGAAT
 -636  CATAATATTC TGAGTTTGGT ATGAAATATT AGTACCATTT AATATTTTTC
 -586  GTATACCGAA TTATTTATGA AGGCATGTAT CGACATGTCC ATTATTCATT
 -536  AGTACAAATT TAAAGAGAAA GTTAAAAGAA TAAATATAAA AAATGTAAAT
 -486  ACATGTCTTT TAGTTGTATC AATTTATTTT TCTTGATATC TTTTTATTTA
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FIG. 4A

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-436  TTAATTTTTT TAAATTGTAC CCTGCATAAA AGAAAATAGA AATAATTGGA
-386  AAAAAAGTAT TATTTTTATA ATACAATCCG ATATAATTAC AATACGATAT
                                                 primer 20
-336  TACCGAATAT TATACTAAAT CAAAATTTAA TTTATCATAT CAAATTATTA
-286  AACTGATATT TCAAATTTTA ATATTTAATA TCTACTTTCA ACTATTATTA
-236  CCTAATTATC AAATGCAAAA TGTATGAGTT ATTTCATAAT AGCCCAGTTC
-186  GTATCCAAAT ATTTTACACT TGACCAGTCA ACTTGACTAT ATAAAACTTT
-136  ACTTCAAAAA ATTAAAAAAA AAAGAAAGTA TATTATTGTA AAAGATAATA
 -86  CTCCATTCAA AATATAAAAT GAAAAAAGTC CAGCGCGGCA ACCGGGTTCC
                              primer 19
                                        +1--->
 -36  TCTATAAATA CATTTCCTAC ATCTTCTCTT CTCCTCACAT CCCATCACTC
 +15  TTCTTTTAAC AATTATACTT GTCAATCATC AATCCCACAA ACAACACTTT
 +65  TTCTCTCCTC TTTTTCCTCA CCGGCGGCAG ACTTACCGGT GAAAAAATGG
+115  ACGTTCGCCG GAGATCTGAA GAGCCTGTTT ATCCATCTAA GGTCTTTGCC
     primer 18   BglII
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FIG. 4B

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+165  GCCGATGAAA AACCTCTCAA ACCCCACAAG AAACAACAAC AACAACAAGA
+215  GGACAAGAAT ACCCTTCTCA TTGATGCTTC CGATGCTCTC CCACTTCCTT
+265  TGTATCTCAC GAATGGCTTG TTTTTTCACCA TGTTTTTCTC TGTTATGTAT
+315  TTTCTTCTAT CAAGGTGGCG TGAGAAAATC AGGAATTC
                                       EcoRI
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FIG. 4C

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   1  CGAATCAACT AGTTATACTC TCTCACTTAT TTTTGTATTT GTACACCTTT
  51  GTCTTACGTG GTGCATTCTG TGTTACTCGA TTGAGGCGCA TGCGAGATAT
 101  TTGGATGCCA AGCCTAAGTC AAAAAGGTGC AGAAAATTAC AAAAAAAAAT
 151  AAAATTCAGA GxGTAATAGG ACGTTAGTTT ATTTAAAGTG TGTTTTTAAA
 201  ATTTCAATTA TAATCTACAA AGATACTTCT GCATTATATA TATATATATA
 251  TATATATATA TATATATATA TATATATATA TATATATATA TATATATATA
 301  TAAAAAGAAG ACAGGTACAT TGGATTTTAG CTTGTTTGGT GTCCCAGCAA
 351  ACAGCXAAGG AGTAGTATTT TAATTAATAA GTAATCAATT TTGGGTGAGA
 401  AATTGCTGAG ATACCTCCTT TGTCTTTTTT TTATATATAT ATATATATAT
 451  ATATATATAT ATATATATAT ATATATATTA
```

FIG.5

EcoRI

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  1  GAATTCGAAG CAACTTtCCT GACTTGTTTG GTCATGCTTA TAGGTTCACC
 51  CAGACTTCGC AGCTCATTTG TAATGGAAGA CAACTTTGTG AACATGTCAT
101  GTATAGTTTC TCCTTCCTTC CTTCATTTTG AAGTTCTCAT ATcGTGAGGT
151  GAGCATGTCa atcTTGGATT CTTTGACTTG TTCAGTTTCT TCATGTGTAG
201  TCAACAAGCA ATCCCAGATT TCTTTAGCAG ACTCACAGGC TGACACTCTA
251  TTGTACTCAT CAGGTCCTAT CCCACAGACC ATAAGAGTTT TACCTTTGAA
301  GCCCTTTTCT ATCTTTTTCC TGTCAGCATC ATCATATTTC TGCTTGGGCT
351  TTGGAaCAGT GATGGTCTTT CTCATCTTtA CTTCATCATT GGACAAGAGT
401  CACTAGTATA ATATCCCATA ACTCGCTATC TTCAGCcATA ATCGTGCATT
451  CTAACTTTCC ACCAACTGTA GAAATGTCCA tTCAAACGAG GAGGTCTATG
501  TGATGACTGA CCTTC
```

FIG.6

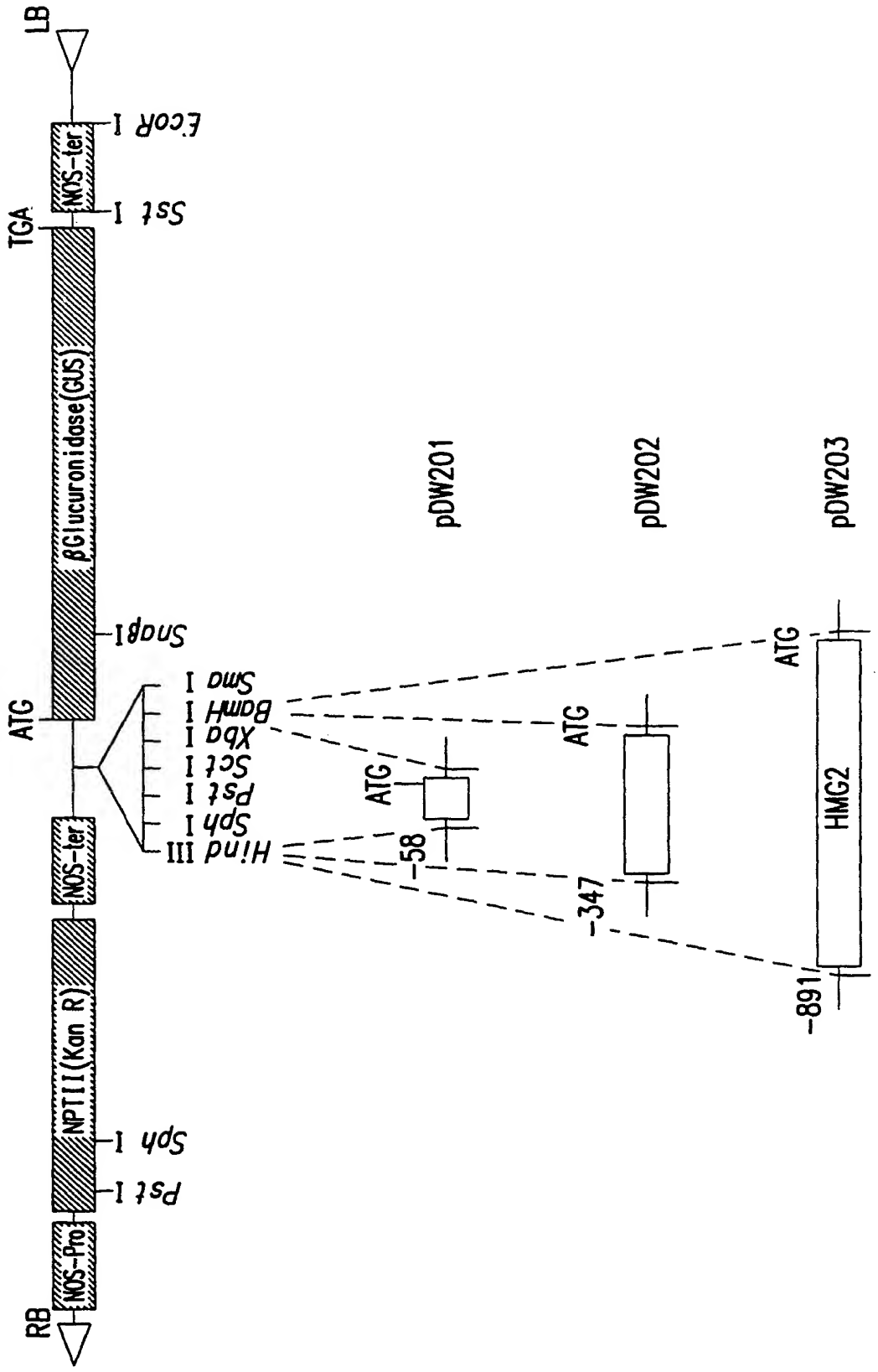

FIG.7

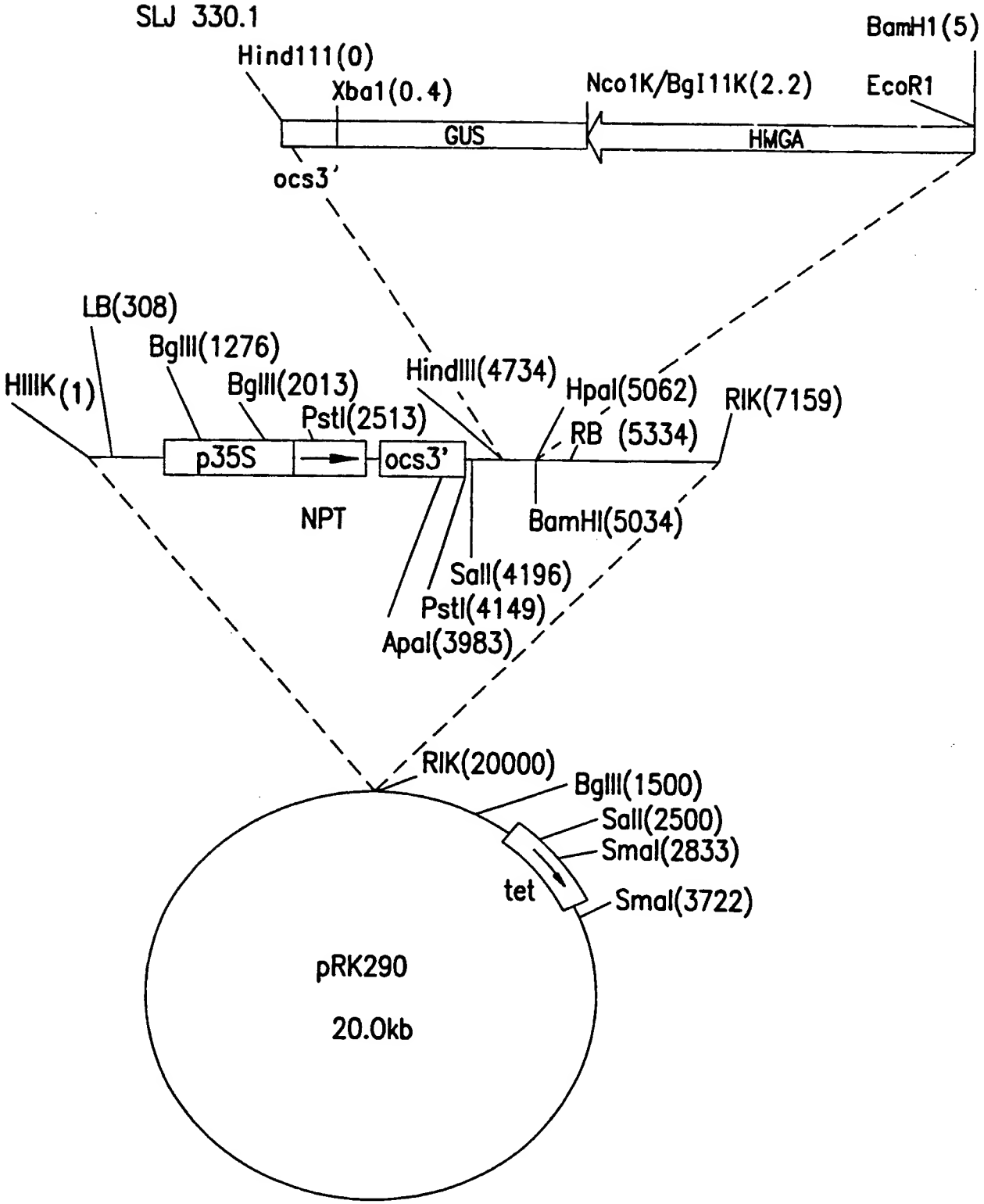

FIG.8

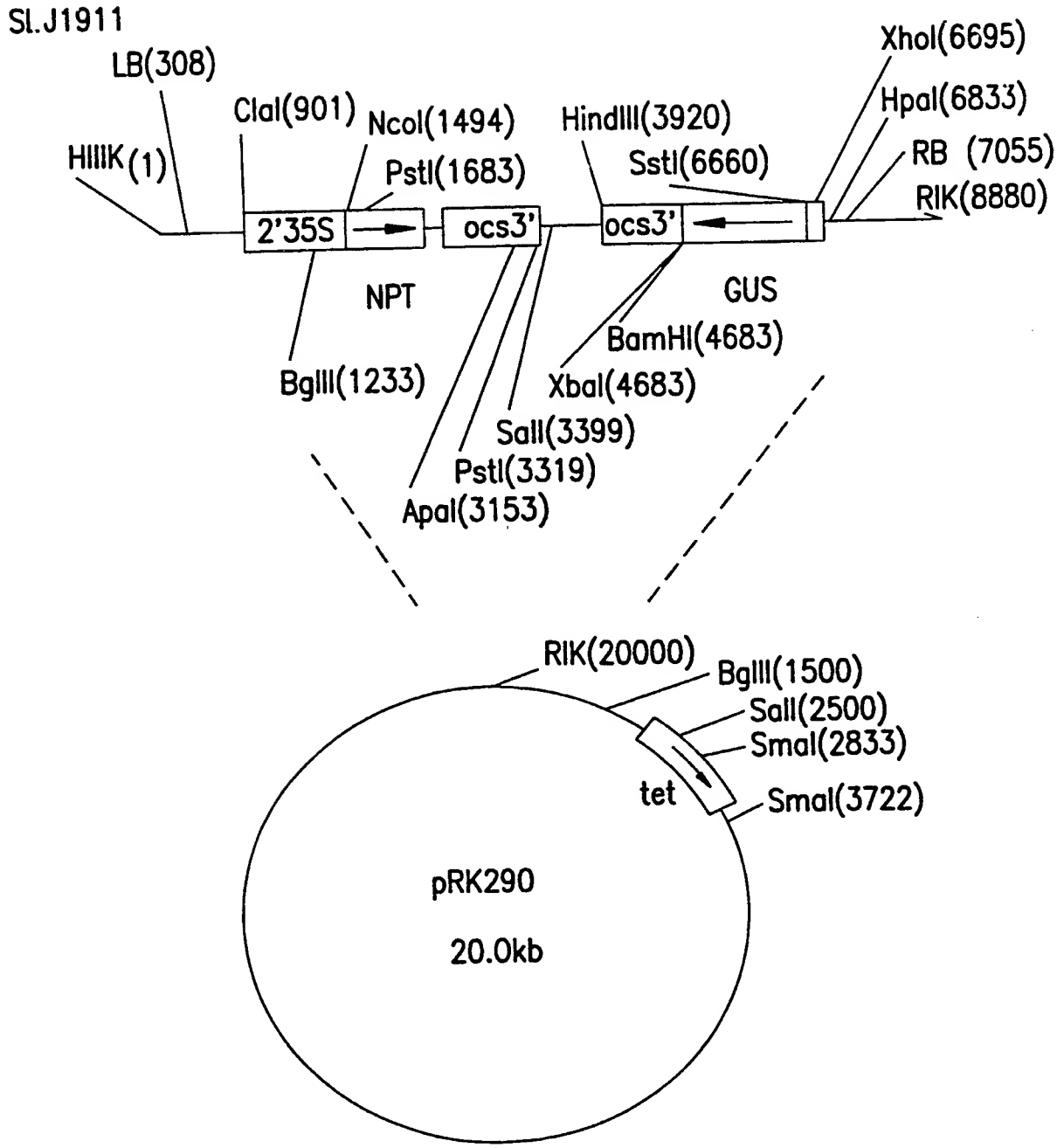

FIG.9

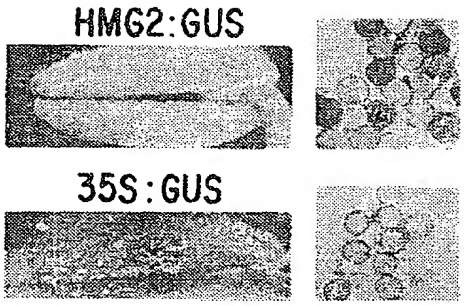

FIG. 10A

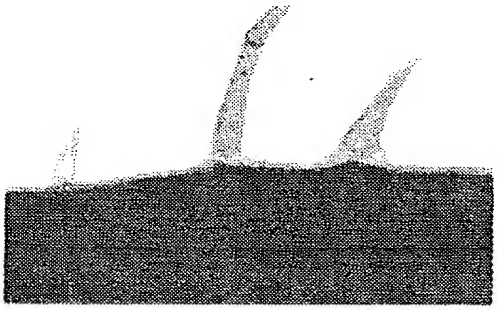

FIG. 10B

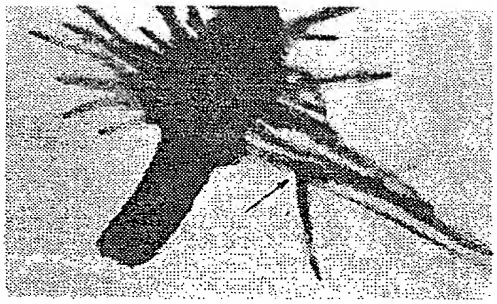

FIG. 10C

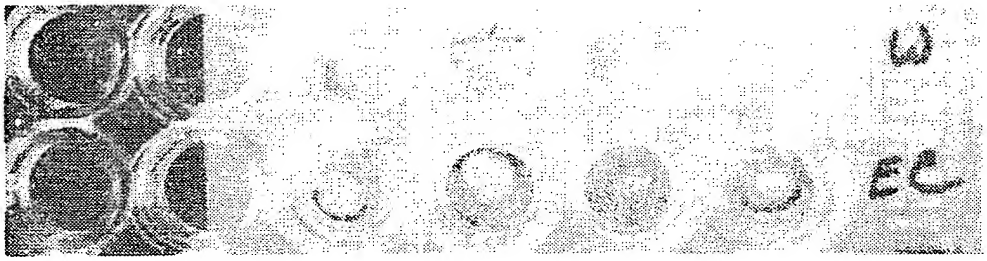

FIG. 11A

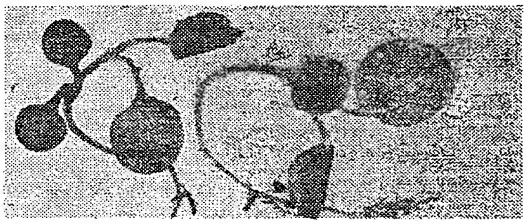

FIG. 11B

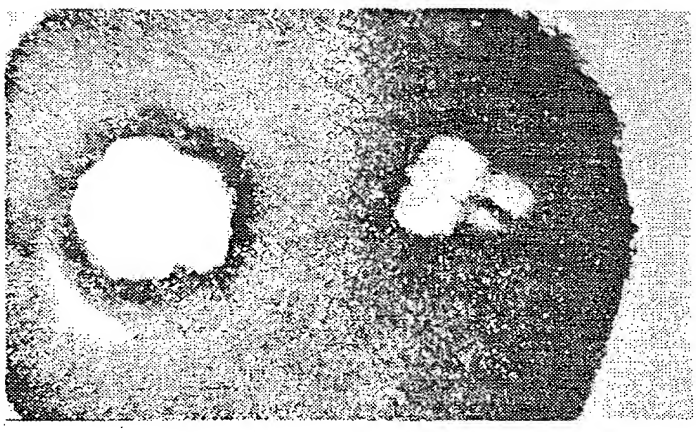

FIG. 11C

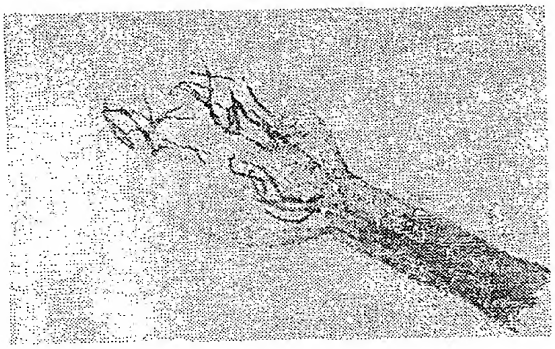

FIG. 12A

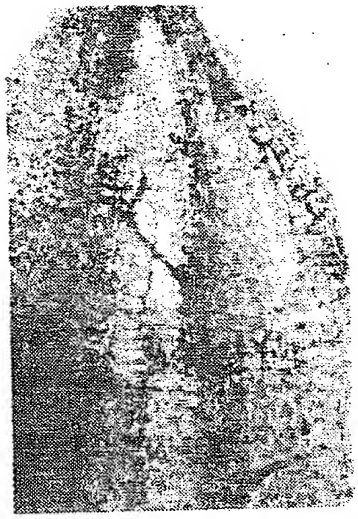

FIG. 12D

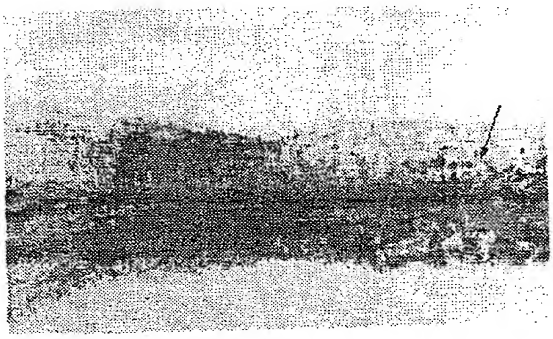

FIG. 12B

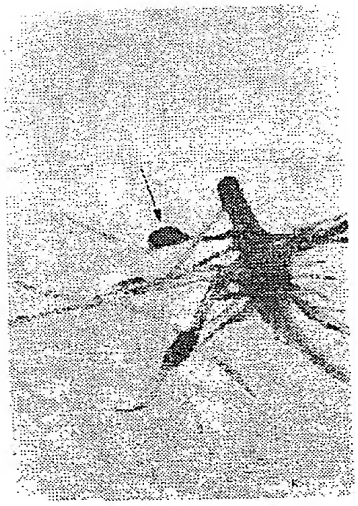

FIG. 12E

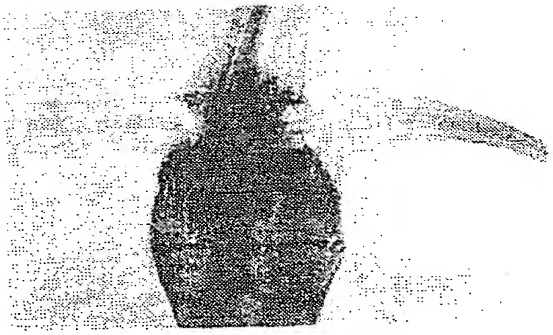

FIG. 12C

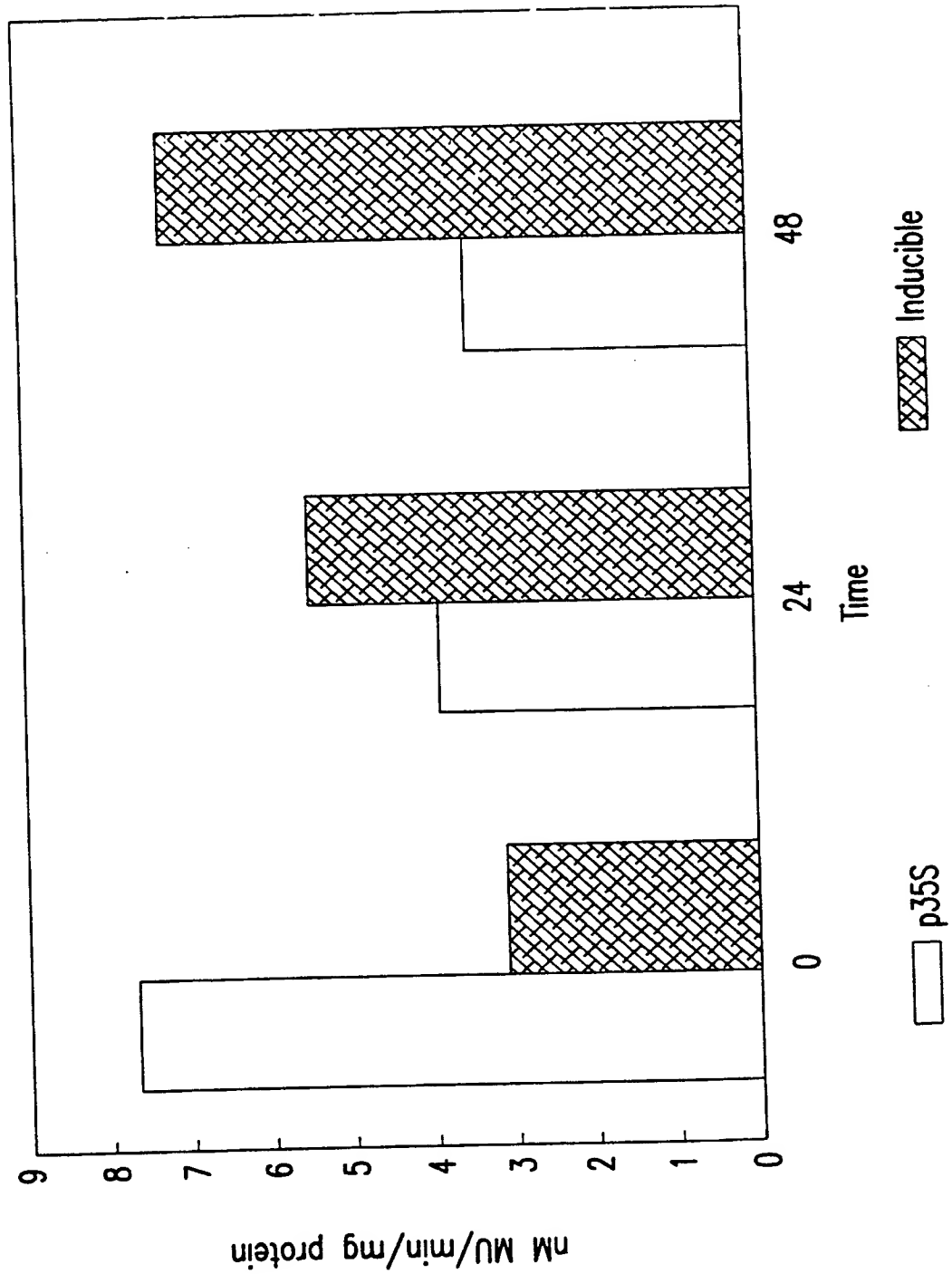

FIG.13

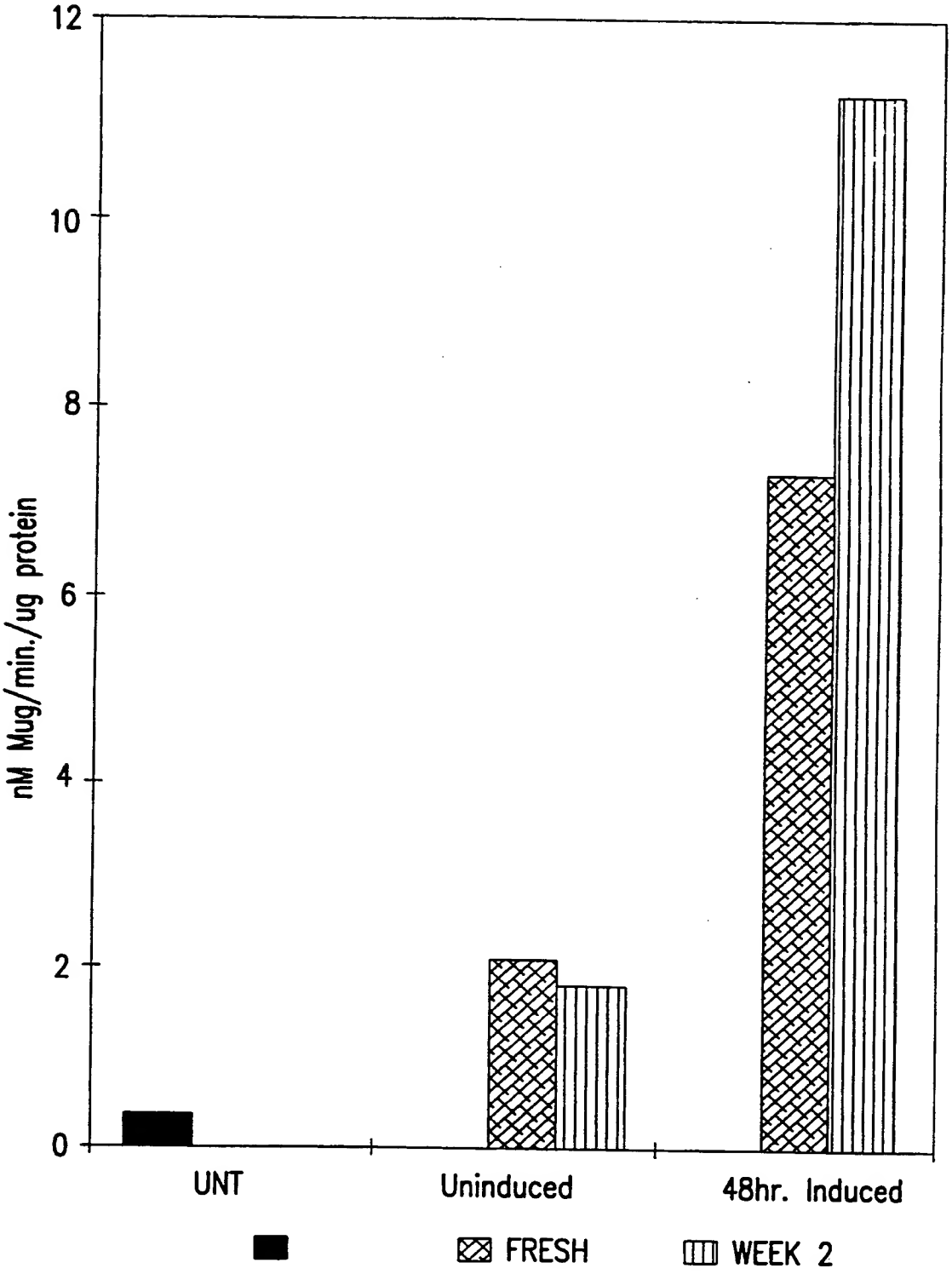
12
10
8
6
4
2
0
nM Mug/min./ug protein
UNT
Uninduced
48hr. Induced
FRESH
WEEK 2

FIG.14

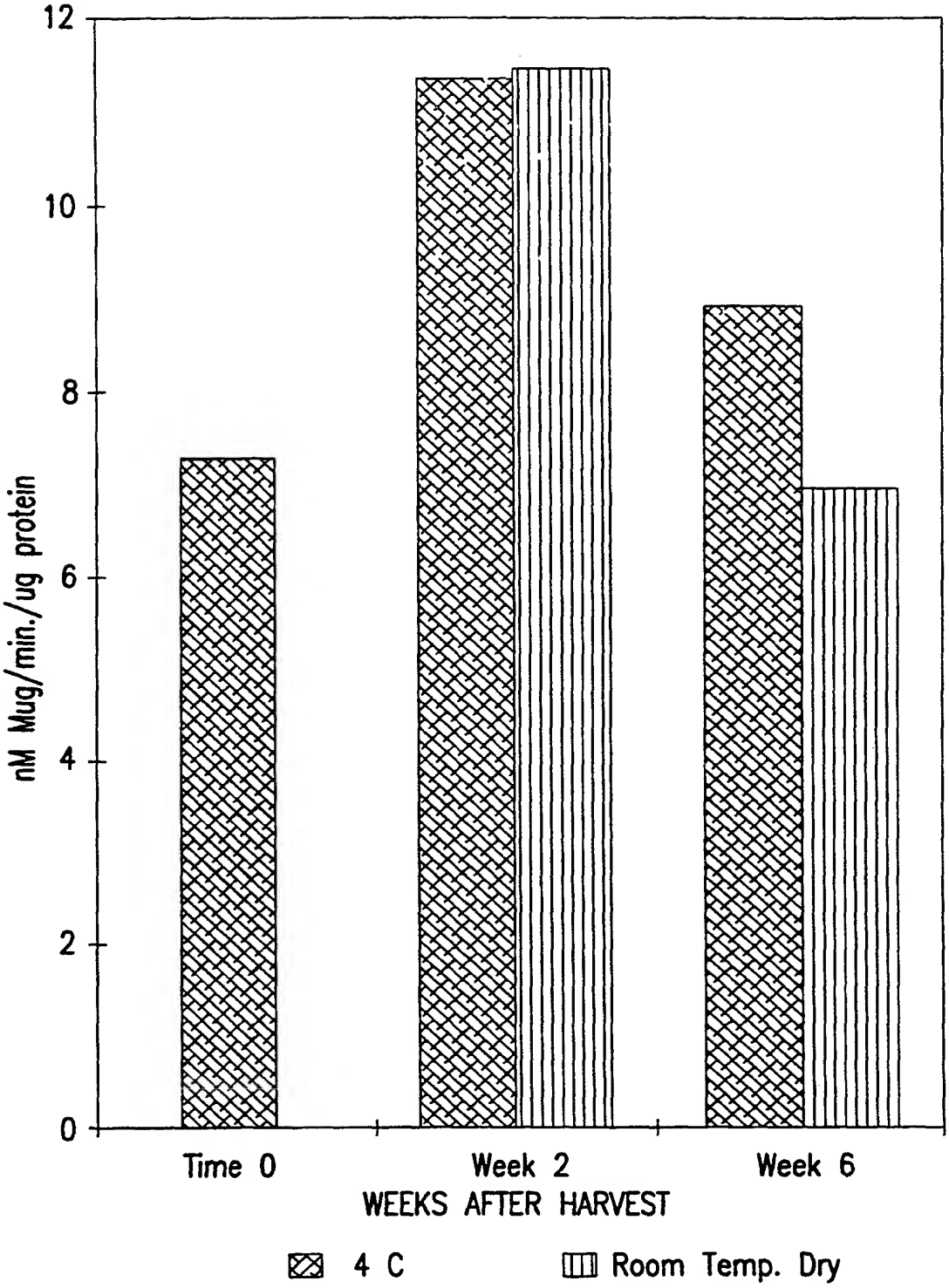

FIG.15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/08722

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A01H 1/04; C12N 15/00; C07H 17/00
US CL :800/205; 435/172.3; 536/24.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/205; 435/172.3; 536/24.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	H. Park, "MOLECULAR CLONING, CHARACTERIZATION AND EXPRESSION OF 3-HYDROXY-3-METHYL GLUTARYL COENZYME A REDUCTASE GENE FROM TOMATO (*LYCOPERSICON ESCULENTUM*, MILL)", Ph.D. dissertation, published 1990 by Virginia Polytechnic Institute and State University, pages 52-74, see page 59.	1-6 ------ 7-38
Y	BIO/TECHNOLOGY, Volume 10, issued May 1992, Williams et al., "Chemical Regulation of *Bacillus Thuringiensis* delta-Endotoxin Expression in Transgenic Plants", pages 539-543, see page 540.	7-28, 30-38

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
27 SEPTEMBER 1994	16 NOV 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer ELIZABETH F. MCELWAIN
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF MOLECULAR BIOLOGY, Volume 266, No. 3, issued January 1991, R. Leah et al., "Biochemical and Molecular Characterization of Three Barley Seed Proteins with Antifungal Properties", pages 1564-1573, see page 1564.	29
Y	PLANT MOLECULAR BIOLOGY, Volume 16, issued 1991, J. M. Casacuberta et al., "A Gene Coding for a Basic Pathogenesis-Related (PR-like) Protein from *Zea mays*. Molecular Cloning and Induction by a Fungus (*Fusarium moniliforme*) in Germinating Maize Seeds", pages 527-536, see page 527.	24-25, 29-30